

Microbial DNA and Host Immunity

Edited by

Eyal Raz, MD



HUMANA PRESS

Microbial DNA and Host Immunity

Microbial DNA and Host Immunity

Edited by

Eyal Raz, MD

*University of California, San Diego
La Jolla, CA*

Humana Press  Totowa, New Jersey

© 2002 Humana Press Inc.
999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

All rights reserved. No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording, or otherwise without written permission from the Publisher.

The content and opinions expressed in this book are the sole work of the authors and editors, who have warranted due diligence in the creation and issuance of their work. The publisher, editors, and authors are not responsible for errors or omissions or for any consequences arising from the information or opinions presented in this book and make no warranty, express or implied, with respect to its contents.

This publication is printed on acid-free paper. (∞)
ANSI Z39.48-1984 (American National Standards Institute) Permanence of Paper for Printed Library Materials.

Production Editors: Tracy Catanese and Diana Mezzina
Cover design by Patricia F. Cleary.

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel.: 973-256-1699; Fax: 973-256-8341; E-mail: humana@humanapr.com or visit our Web site: <http://humanapress.com>

Photocopy Authorization Policy:

Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Humana Press Inc., provided that the base fee of US \$10.00 per copy, plus US \$00.25 per page, is paid directly to the Copyright Clearance Center at 222 Rosewood Drive, Danvers, MA 01923. For those organizations that have been granted a photocopy license from the CCC, a separate system of payment has been arranged and is acceptable to Humana Press Inc. The fee code for users of the Transactional Reporting Service is: [0-58829-022-0/02 \$10.00 + \$00.25].

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

Microbial DNA and host immunity / edited by Eyal Raz

p. ; cm.

Includes bibliographical references and index.

ISBN 1-58829-022-0 (alk. paper)

[DNLM: 1. DNA, Bacterial--immunology. 2. Adjuvants, Immunologic. 3. Vaccines.

QW 52 M6238 2003]

Qr186.6 B33 M535 2003

616'.014--dc21

2002068938

Dedication

In memory of my dear parents, Miriam and Chaim Raz.

Preface

The observation that bacterial DNA, or its synthetic immunostimulatory oligonucleotide (ISS-ODN) analogs containing unmethylated CpG dinucleotide, are potent activators of innate immunity has attracted a wide spectrum of scientists with interest in basic and/or translational research. Like other microbial products, e.g., peptidoglycans, lipopeptides, lipopolysaccharides, or dsRNA, bacterial DNA triggers its immune properties via a member of the TLR family (i.e., TLR9). These immune properties are aimed mainly at providing an immediate defense mechanism in the mammalian host. Bacterial DNA stimulates the production of type-1 cytokines such as IL-12 and IFNs, and enhances the expression of various co-stimulatory ligands such as B7, CD40, and ICAM-1, as well as class I and class II MHC molecules, mainly by and on antigen presenting cells. This wide range of activities contributes to the use of ISS-ODN as a unique adjuvant that induces both Th1 and CTL responses to experimental and relevant clinical antigens. To date, ISS-ODN has been used as an adjuvant in a variety of clinical trials in the fields of infectious disease, allergy, and cancer. Recent data also identified unique immunomodulating properties and antiinflammatory activities induced by ISS-ODN in an antigen-independent fashion. These inhibit allergic inflammation and colitis in various animal models, respectively.

Based on the progress made to date in uncovering the basic biological principles of immune activation by immunostimulatory DNA and the initial encouraging data emerging from related clinical trials, it is predicted that more efforts will be invested in this field by both academia and industry. It is anticipated that in next few years, our knowledge of this area will be further expanded and that potentially important applications derived from this understanding will find their way to various aspects of clinical medicine.

I wish to thank all the authors and their colleagues for their contributions, the editors for their help and support, and Jane Uhle for her determination and help in putting this book together in a relatively short time.

Eyal Raz, MD

Contents

Preface	vii
Contributors	xiii

PART I: INTRODUCTION

1 Immunostimulatory DNA: <i>An Overview</i> <i>Eyal Raz</i>	3
2 Historical Perspectives <i>Saburo Yamamoto, Toshiko Yamamoto, and Tohru Tokunaga</i>	9

PART II: RECEPTORS AND SIGNALING

3 Signal Transduction Pathways Activated By CpG-DNA <i>Hans Häcker</i>	17
4 A Novel Toll-Like Receptor that Recognizes Bacterial DNA <i>Hiroaki Hemmi and Shizuo Akira</i>	39
5 Activation of Innate Immunity by Microbial Nucleic Acids <i>Wen-Ming Chu, Xing Gong, and Tony Yoon</i>	49
6 Phosphorothioate Backbone Modification Changes the Pattern of Responses to CpG <i>Katryn J. Stacey, David P. Sester, Shalin Naik, Tara L. Roberts, Matthew J. Sweet, and David A. Hume</i>	63

PART III: CELL ACTIVATION

7 Activation of NK Cell By Immunostimulatory Oligo-DNA in Mouse and Human <i>Saburo Yamamoto, Toshiko Yamamoto, Tetsuro Kataoka, Sumiko Iho, and Tohru Tokunaga</i>	81

- 8 Regulation of Antigen Presenting Cell Function
by CpG DNA
*David Askew, Rose S. Chu,
and Clifford V. Harding* 91
- 9 Activation of B Cells by CpG Motifs in Bacterial DNA
Ae-Kyung Yi and Arthur M. Krieg 103
- 10 IFN-Dependent Pathways for Stimulation of Memory
CD8⁺ Cells
Jonathan Sprent 129
- 11 Cross-Priming of CD8⁺ T Cells by Immunostimulatory
Sequence DNA
Hearn Jay Cho, Sandip Datta, and Eyal Raz 137

PART IV: VACCINATION STRATEGIES

- 12 The Th1 Adjuvant Effect of Immunostimulatory (ISS)
DNA Sequences
Maripat Corr and Chih Min Tang 153
- 13 Immunostimulatory DNA Prepriming for the Induction
of Th1 and Prevention of Th2 Biased
Immune Responses
*Hiroko Kobayashi, Elena Martin-Orozco,
Kenji Takabayashi, and Anthony A. Horner* 163
- 14 Protein-Immunostimulatory DNA-Conjugate:
A Novel Immunogen
*Kenji Takabayashi, Helen Tighe, Lucinda Beck,
and Hans L. Spiegelberg* 175
- 15 Immunostimulatory DNA Sequence-Based Mucosal
Vaccines
Anthony A. Horner 189
- 16 Enhancement of the Immunoadjuvant Activity
of Immunostimulatory DNA Sequence
by Liposomal Delivery
*Eli Kedar, Igal Louria-Hayon, Aviva Joseph,
Zichria Zakay-Rones, Tomoko Hayashi, Kenji
Takabayashi, and Yechezkel Barenholz* 203
- 17 Immunostimulatory Sequences in Plasmid Vectors
*Christina C. N. Wu, Chih Min Tang,
Brian Crain, and Maripat Corr* 219

PART V: APPLICATIONS—INFECTIOUS DISEASE

- 18 Comparison of CpG DNA with Other Adjuvants
for Vaccination Against Hepatitis B
*Michael J. McCluskie, Risini D. Weeratna,
and Heather L. Davis* 229
- 19 Immunostimulatory DNA-Based Immunization:
Hope for an HIV Vaccine?
*Sandip K. Datta, Anthony A. Horner,
Kenji Takabayashi, and Eyal Raz* 239
- 20 Immunoprotective Activity of CpG Oligonucleotides
*Dennis M. Klinman, Ken J. Ishii,
and Ihsan Gursel* 255
- 21 Protective Immunity of Immunostimulatory Sequences
Against Mycobacterial Infection
*Tomoko Hayashi, Christine H. Tran,
Lucinda Beck, Savita Rao,
and Antonino Cantanzaro* 265

PART VI: APPLICATIONS—ALLERGY

- 22 DNA-Based Immunotherapeutics For Allergic Disease
Anthony A. Horner and Eyal Raz 279
- 23 Immunostimulatory DNA for Allergic Asthma
*Reid K. Ikeda, Kenji Takabayashi,
and David Broide* 289
- 24 CpG Oligodeoxynucleotides in Asthma
Kunihiko Kitagaki and Joel N. Kline 301
- 25 Modulation of Allergic Conjunctivitis
by Immunostimulatory DNA Sequence
Oligonucleotides
Andrea Keane-Myers and Chi-Chao Chan 315

PART VII: APPLICATIONS—CANCER

- 26 CpG Oligodeoxynucleotides and Monoclonal
Antibody Therapy of Lymphoma
Thomas L. Warren and George J. Weiner 329

PART VIII: INFLAMMATION AND AUTOIMMUNITY

- 27 The Antigenicity of Bacterial DNA
David S. Pisetsky 341

28	Inflammatogenic Properties of Immunostimulatory DNA Sequences <i>Andrej Tarkowski, L. Vincent Collins, Guo-Min Deng, and Leno Mölne</i>	351
29	The Role of Immunostimulatory DNA Sequences in Arthritis <i>Sarah T.A. Roord, Arash Ronaghy, Berent J. Prakken, Kenji Takabayashi, and Dennis A. Carson</i>	363
30	Effects of Immunostimulatory DNA Oligonucleotides on Experimental Colitis <i>Daniel Rachmilewitz, Fanny Karmeli, Leonor Leider-Trejo, Kenji Takabayashi, Tomoko Hayashi, and Eyal Raz</i>	373
PART IX: SAFETY CONSIDERATIONS		
31	CpG ODN—Safety Considerations <i>Daniela Verthelyi and Dennis M. Klinman</i>	385
Index		397

Contributors

- SHIZUO AKIRA • *Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
- DAVID ASKEW • *Department of Pathology, Case Western Reserve University, Cleveland, OH*
- LUCINDA BECK • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- DAVID H. BROIDE • *Department of Medicine, University of California at San Diego, La Jolla, CA*
- DENNIS A. CARSON • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- ANTONINO CATANZARO • *Department of Medicine, University of California San Diego, San Diego, CA*
- CHI-CHAO CHAN • *Immunopathology Section, Laboratory of Immunology, NIH, Bethesda, MD*
- HEARN JAY CHO • *Division of Hematology/Oncology, New York Presbyterian Hospital, New York, NY*
- ROSE S. CHU • *Department of Pathology, Case Western Reserve University, Cleveland, OH*
- WEN-MING CHU • *Department of Molecular Microbiology and Immunology, Brown University, Providence, RI*
- VINCENT COLLINS • *Department of Rheumatology and Inflammation Research, Göteborg University, Göteborg, Sweden*
- MARIPAT CORR • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- BRIAN CRAIN • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*

- SANDIP K. DATTA • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- HEATHER L. DAVIS • *Coley Pharmaceutical Canada, Loeb Health Research Institute, and Faculties of Health Sciences and Medicine, University of Ottawa, Ottawa, Canada*
- GUO-MIN DENG • *Department of Rheumatology and Inflammation Research, Göteborg University, Göteborg, Sweden*
- XING GONG • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- IHSAN GURSEL • *Center for Biologics Evaluation & Research, Food and Drug Administration, Department of Health and Human Services, Bethesda, MD*
- HANS HÄCKER • *Institute of Medical Microbiology, Immunology and Hygiene, Munich, Germany*
- CLIFFORD V. HARDING • *Department of Pathology, Case Western Reserve University, Cleveland, OH*
- TOMOKO HAYASHI • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- HIROAKI HEMMI • *Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
- ANTHONY A. HORNER • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- DAVID A. HUME • *Institute for Molecular Bioscience and Departments of Microbiology and Biochemistry, University of Queensland, Brisbane, Australia*
- SUMIKO IHO • *Fukui Medical University, Fukui, Japan*
- REID K. IKEDA • *Department of Medicine, University of California San Diego, La Jolla, CA*
- KEN J. ISHII • *Center for Biologics Evaluation & Research, Food and Drug Administration, Department of Health and Human Services, Bethesda, MD*
- FANNY KARMELI • *Division of Medicine, Shaare Zedek Medical Center, Jerusalem, Israel*

- TETSURO KATAOKA • *National Institute of Infectious Diseases and Biomedical Science Association, Tokyo, Japan*
- ANDREA KEANE-MYERS • *Laboratory of Allergic Disease, NIAID/NIH, Bethesda, MD*
- ELI KEDAR • *Department of Immunology, Hebrew University, Hadassah Medical School, Jerusalem, Israel*
- KUNIIHIKO KITAGAKI • *Division of Pulmonary Medicine, University of Iowa Health Center, Iowa City, IA*
- JOEL KLINE • *Division of Pulmonary Medicine, University of Iowa Health Center, Iowa City, IA*
- DENNIS M. KLINMAN • *Center for Biologics Evaluation & Research, Food and Drug Administration, Department of Health and Human Services, Bethesda, MD*
- HIROKO KOBAYASHI • *Department of Internal Medicine II, Fukushima Medical University School of Medicine, Fukushima, Japan*
- ARTHUR M. KRIEG • *Division of Pulmonary Medicine, University of Iowa Health Center, Iowa City, IA*
- LEONOR LEIDER-TREJO • *Department of Pathology, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel*
- ELENA MARTIN-OROZCO • *Departamento de Neuroquímica, Universidad Miguel Hernandez, Campus de San Juan, San Juan, Alicante, Spain*
- MICHAEL J. MCCLUSKIE • *Coley Pharmaceutical Canada, Ottawa, Canada*
- LENA MÖLNE • *Department of Rheumatology and Inflammation Research, Göteborg University, Göteborg, Sweden*
- SHALIN NAIK • *Department of Microbiology, University of Queensland, Brisbane, Australia*
- DAVID S. PISETSKY • *Division of Rheumatology, Department of Medicine, VA Medical Center, Durham, NC*
- BERENT J. PRAKKEN • *Department of Pediatrics, University Medical Center, Wilhelmina Children's Hospital, Utrecht, The Netherlands, and Department of Pediatrics, University of California at San Diego, La Jolla, CA*
- DANIEL RACHMILEWITZ • *Division of Medicine, Shaare Zedek Medical Center, Jerusalem, Israel*
- SAVITA RAO • *Department of Medicine, University of California San Diego, San Diego, CA*
- EYAL RAZ • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*

- TARA L. ROBERTS • *Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia*
- ARASH RONAGHY • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- SARAH T. A. ROORD • *Department of Pediatrics, University Medical Center, Wilhelmina Children's Hospital, Utrecht, The Netherlands, and Department of Pediatrics, University of California at San Diego, La Jolla, CA*
- DAVID P. SESTER • *Department of Microbiology, University of Queensland, Brisbane, Australia*
- HANS L. SPIEGELBERG • *Department of Pediatrics, University of California at San Diego, La Jolla, CA*
- JONATHAN SPRENT • *Department of Immunology, The Scripps Research Institute, La Jolla, CA*
- KATRYN STACEY • *Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia*
- MATTHEW J. SWEET • *Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia*
- KENJI TAKABAYASHI • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- CHIH MIN TANG • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- ANDREJ TARKOWSKI • *Department of Rheumatology, Göteborg University, Göteborg, Sweden*
- HELEN TIGHE • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*
- TOHRU TOKUNAGA • *Fukuoka Woman's University, Fukuoka, Japan*
- CHRISTINE H. TRAN • *Division of Rheumatology, Allergy and Immunology, and The Sam and Rose Stein Institute for Research on Aging, Department of Medicine, University of California at San Diego, La Jolla, CA*

- DANIELA VERTHELYI • *Center for Biologics Evaluation & Research,
Food and Drug Administration, Department of Health and
Human Services, Bethesda, MD*
- THOMAS L. WARREN • *Holden Comprehensive Cancer Center,
University of Iowa, Iowa City, IA*
- RISINI D. WEERATNA • *Coley Pharmaceutical Canada, Ottawa, Canada*
- GEORGE WEINER • *Holden Comprehensive Cancer Center, University of
Iowa, Iowa City, IA*
- CHRISTINA C. N. WU • *Division of Rheumatology, Allergy and
Immunology, and The Sam and Rose Stein Institute for Research
on Aging, Department of Medicine, University of California at
San Diego, La Jolla, CA*
- SABURO YAMAMOTO • *National Institute of Infectious Diseases,
Musashimarayama, Tokyo, Japan*
- TOSHIKO YAMAMOTO • *National Institute of Infectious Diseases,
Musashimarayama, Tokyo, Japan*
- AE-KYUNG YI • *Children's Foundation Research Center at Le Bonheur
Children's Hospital and Department of Pediatrics, University
of Tennessee Health Science Center, Memphis, TN*
- TONY YOON • *Division of Rheumatology, Allergy and Immunology,
and The Sam and Rose Stein Institute for Research on Aging,
Department of Medicine, University of California at San Diego,
La Jolla, CA*

PART I

INTRODUCTION

Immunostimulatory DNA

An Overview

Eyal Raz

1. IMMUNOSTIMULATORY DNA: AN ACTIVATOR OF INNATE IMMUNITY

Immunostimulatory DNA sequences (ISS or CpG motif) were first used as an adjuvant over 60 years ago. Whole mycobacterial extract mixed with paraffin oil constitutes Freund's famous adjuvant (*see* Chapter 2). However, mycobacterial and other bacterial DNAs were shown to contribute to the adjuvanticity of this complex reagent only in the early 1980s. Tokunaga and coworkers discovered that DNA purified from *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) induced limited anti-tumor activity. This BCG DNA mediated anti-tumor activity was shown to produce various cytokines (e.g., IFN α/β , IL-12, and IL-6) and to activate a variety of immunocytes, such as natural killer (NK) cells (*see* Chapter 7), B cells (*see* Chapter 9) macrophages and dendritic cells (*see* Chapter 8). These activities were eliminated by DNase treatment. In subsequent studies, the ISS were found to contain unmethylated CpG dinucleotides within a given hexamer that followed the formula: 5'-purine-purine-CG-pyrimidine-pyrimidine-3' (e.g., 5'-GACGTC-3') or 5'-purine-TCG-pyrimidine-pyrimidine-3' (e.g., 5'-GTCGTC-3'). Interestingly, CpG dinucleotides generally occur at or near the expected frequency, 1 in 16, in many prokaryotic genomes but are much less frequent in eukaryotic DNA. In addition, less than 5% of the cytosines in prokaryotic CpG dinucleotides are methylated, whereas 70–90% of the CpG dinucleotides in eukaryotic genomes contain an inactivating methylated cytosine. These observations have led to the proposal that the immune system has evolved the ability to detect CpG motifs found in bacterial genomes as danger signals, stimulating an innate immune response in mammals in anticipation of impending microbial infection. Similar arguments were made previously for other microbial products such as LPS or dsRNA.

2. INNATE IMMUNITY AND TOLL LIKE RECEPTORS (TLR)

The innate immune system expresses a variety of pattern recognition receptors that discriminate between pathogens and the host. These receptors interact with microbial components, which are conserved among a wide variety of microorganisms and are termed pathogen-associated molecular patterns (PAMPs) (1). By interacting with various PAMPs, the Toll like receptor (TLR) family has been recently identified as crucial for the recognition of microbial infection. The prototypic TLR in *Drosophila* was first identified in connection with developmental events and later in connection with the production of antimicrobial peptides with antifungal and antibacterial activity (1). This later finding promoted an intensive search for its mammalian analog(s). The mammalian TLR has been phylogenetically conserved and is characterized as a type I transmembrane protein with a Toll/IL-1R homology (TIR) in its cytoplasmic domain (2). The microbial ligands of the 10 mammalian TLRs are partially known. TLR2 interacts with peptidoglycan, bacterial lipopeptides, and certain species of LPS (3). TLR3 binds to dsRNA (4), TLR4 interacts with LPS (5) and TLR5 recognizes bacterial flagellin (6). TLR9 was shown to recognize ISS (*see* Chapter 4). Certain TLRs (i.e., TLR1, TLR2 and TLR6) are also recruited into the macrophage phagosome where they are heterodimerized, interact with their microbial ligands, and cause cell activation (7). This cooperation among certain TLRs expands the ligand repertoire of the TLR family to discriminate among the large number of PAMPs found in the outside environment. Recent studies have also proposed that host-derived factors (8) and HSP60 (9) were implicated as ligands for TLR4. The physiological consequence of this later interaction may have to do with the previously proposed “danger” like signal, which alerts the host immune system to the presence of foreign antigen via the release of various products originating from its own damaged tissues (10).

Activation of immune cells by bacterial DNA has been shown to require cellular uptake of the DNA by DNA sequence-independent endocytosis, and later, by endosomal acidification (11). The required acidification in mediating signaling by bacterial DNA is unique for TLR9 signaling. This finding supports the assumption that certain TLRs are recruited into the phagosome where they are activated by their ligands released from the lysed pathogen (7) and further suggests that different TLR ligands induce cell activation at various stages of endosomal maturation.

The interaction of a TLR with its ligand induces recruitment of a signaling complex, which includes MyD88, IRAK, and TRAF6 to the TIR domain (*see* Chapter 3). The cytoplasmic organization of this signaling complex

leads to activation of NF- κ B and MAPK pathways and to the subsequent gene transcription of cytokines (e.g., IL-6 and IL-12) and costimulatory molecules (e.g., CD40 and B7). Based on this scheme, any TLR-TLR ligand interaction should result in an identical activation profile. However, this is not the case as substantial differences are observed in the activation profile induced by different TLR ligands (12). It is believed that additional collateral pathways, downstream to the TLR, are responsible for the specificity of each TLR-mediated activation profile (12,2). This assumption was recently supported by the identification of other adaptor proteins such as TIRAP (13), which interact with the TIR domain of TLR4. Thus, one of the future challenges in studying TLR-mediated cell activation is to identify the unique molecular pathway downstream to each TLR that results in a distinctive gene profile from other related pathways in the TLR family.

3. IMMUNOSTIMULATORY DNA, TLR9, AND DNA-PK

TLR9 was recently shown to be required for ISS-induced cell activation (*see* Chapter 4). The cloning and characterization of the mouse and human TLR9 may explain differences in ISS sequences, which optimally bind to mouse or human TLR9. As only approx 75% of the amino acids in the mouse TLR9 are present in human TLR9, differences in the TLR-9 amino acid sequences could contribute to the specificity of recognition of ISS. TLR9-deficient dendritic cells did not demonstrate any response to ISS-ODN *in vitro* and *in vivo*. Activation of signaling cascades mediated by ISS-ODN (MAPK and NF- κ B) is impaired in TLR9-deficient dendritic cells and is restored by genetic complementation using hTLR9 cDNA transfectoma (14,15). In addition, TLR9-deficient mice are resistant to ISS-mediated lethal shock and did not mount a Th1-like response upon immunization with antigen mixed with ISS-ODN (*see* Chapter 4). These data demonstrate that TLR9 plays a critical role in both ISS-mediated cell activation and induction of immune responses. It was recently demonstrated that *in vivo* administration of bacterial-DNA or ISS-ODN to mice lacking the catalytic subunit of DNA-PK (DNA-dependent protein kinase) i.e., DNA-PKcs, and *in vitro* ISS-ODN stimulation of BMDM from these mice results in defective induction of IL-6 and IL-12 (*see* Chapter 5). Further analysis using BMDM from IKKb^{-/-} mice revealed that both DNA-PKcs and IKKb are essential for normal cytokine production in response to ISS-ODN or bacterial-DNA in that ISS-ODN and bacterial-DNA activate DNA-PK, which in turn contributes to the activation of IKKb of NF- κ B. The relationship between TLR9 and DNA-PKcs in mediating ISS-induced cell activation is still unclear. A potential clue for such an interaction was recently described.

TIRAP, a MyD88 analog, was shown to interact with PKR (protein kinase of dsRNA) to mediate LPS signaling (13). The dependence of ISS-mediated signaling on MyD88 (but not on TIRAP) may suggest that MyD88 interacts with DNA-PK to mediate ISS signaling.

4. TLR- AND MYD88-INDEPENDENT CELL ACTIVATION BY MICROBIAL TLR LIGANDS

Although TLR4 is considered to be essential for LPS-induced cell activation (5), two other molecules, Nod1 and Nod2, were recently identified to mediate LPS cell activation. These cytoplasmic molecules contain an N-terminal caspase recruitment domain, a nucleotide-binding domain and C terminal leucine rich repeat (LRR), the latter of which was shown to bind LPS. Transfection of cells that are nonresponsive to LPS with Nod1 or Nod2 cDNA restores their LPS responsiveness and suggests a TLR4-independent pathway for LPS signaling (16). As was mentioned previously, the binding of TLR to its ligand recruits a signaling complex to the cytoplasmic TIR domain (2) that includes MyD88, IRAK, and TRAF6. This signaling complex leads to activation of NF- κ B and MAPK pathways and to subsequent gene transcription. However, recent work has demonstrated that MyD88-deficient but not TLR4-deficient dendritic cells undergo maturation after being stimulated with LPS (17). The MyD88-deficient dendritic cells could upregulate the expression of the B7 and CD40 costimulatory molecules and could efficiently stimulate naive T cells. The molecular pathway behind MyD88-independent activation by LPS was recently uncovered. TIRAP (or Mal) is a MyD88 analog that can mediate LPS signaling in the absence of MyD88 (13). Taken together, these observations indicate the presence of a TLR4-independent activation pathway (16) as well as a TLR4-dependent but MyD88-independent activation pathway (17) for LPS signaling. Thus, the presence of Nod1/Nod2 provides the molecular basis for an alternative activation pathway for microbial TLR ligands while the presence of additional TIR-related accessory proteins provides the molecular basis for collateral activation pathways. The presence of accessory pathways may therefore explain the different activation profiles observed for different TLR ligands (18).

5. APPLICATIONS OF ISS

The immunological properties of ISS represent an emerging field in immuno-biology, which has attracted the interest and attention of scientists in academic institutes and in biotech-based industries. The potential applications of ISS are mainly related, but not limited, to vaccination and to immunotherapy. Indeed, ISS, usually used as synthetic phosphorothioate

ODN, was proven to be an effective Th1, CTL (via cross-priming) and mucosal adjuvant, generating immune responses to coadministered protein antigens which mimic the immune responses seen after gene vaccination. In fact, the Th1 adjuvant activity (*see* Chapter 12 Corr et al.) and the CTL adjuvant activity (*see* Chapter 11 Cho et al.) elicited by ISS have prompted the use of ISS as antiallergic vaccine (*see* Chapter 15 Horner et al.) and antimicrobial vaccine adjuvants (*see* Chapter 18 McCluskie et al.), respectively.

To date ISS, has been used as an adjuvant with a variety of clinically relevant antigens and by different immunization schemes. These include:

1. ISS antigen co-delivery with or without liposomes (*see* Chapter 16).
2. ISS delivery prior to antigen administration (prepriming, *see* Chapter 13).
3. Delivery of ISS-ODN conjugated to the antigen of interest (*see* Chapter 14).
4. In the case of gene vaccination, by codelivery or by subcloning ISS to the immunization vector (*see* Chapter 17).

ISS was also shown to mediate potent immunomodulatory properties, which were promising in a variety of models of experimental allergy (*see* Chapter 23), experimental colitis (*see* Chapter 30) and in models of cancer (*see* Chapter 26).

Several potential safety concerns regarding the use of ISS were addressed in animal models and will need to be addressed in current and future human studies. Studies with ISS will carefully need to monitor the induction of autoantibodies especially against DNA and that the Th1 adjuvanticity of ISS does not induce or exacerbate Th1-mediated diseases (*see* Chapter 31).

Finally, ISS-based clinical trials for infectious and allergic diseases are in progress and have already provided some useful information and generated cautious optimism that the data generated in animal models can be translated to humans.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI-40682 and AI-47078, and by a grant from Dynavax Technologies Corporation.

REFERENCES

1. Medzhitov, R. and Janeway, C. Jr. (2000) The Toll receptor family and microbial recognition. *Trends Microbiol.* **10**, 452–456.
2. Akira, S., Takeda, K., and Kaisho, (2001) T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**, 675–680.
3. Werts, C., Tapping, R. I., Mathison, J. C., et al. (2001) R.J. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat. Immunol.* **2**, 346–352.

4. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732–738.
5. Beutler, B. (2000) Endotoxin, toll-like receptor 4, and the afferent limb of innate immunity. *Curr. Opin. Microbiol.* **3**, 23–28.
6. Hayashi, F., Smith, K. D., Ozinsky, A., et al. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099–1103.
7. Ozinsky, A., Underhill, D. M., Fontenot, J. D., et al. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl. Acad. Sci. USA.* **97**, 13,766–13,771.
8. Okamura, Y., Watari, M., Jerud, E. S., et al. (2001) The extra domain A of fibronectin activates Toll-like receptor 4. *J. Biol. Chem.* **276**, 10,229–10,233.
9. Ohashi, K., Burkart, V., Flohe, S., and Kolb, H. (2000) Heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J. Immunol.* **164**, 558–561.
10. Gallucci, S. and Matzinger, P. (2001) Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* **13**, 114–119.
11. Yi, A. K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J., and Krieg, A.M. (1998) CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* **160**, 4755–4761.
12. Jones, B. W., Means, T. K., Heldwein, K. A., et al. (2001) Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukoc. Biol.* **69**, 1036–1044.
13. Horng, T., Barton, G.M., and Medzhitov, R. (2001) TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol.* **2**, 835–841.
14. Bauer, S., Kirschning, C. J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G. B. (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA.* **98**, 9237–9242.
15. Takeshiata, F., Leifard, C.A., Gursel, I., et al. (2001) Role of TLR9 in CpG DNA-induced activation of human cells. *J. Immunol.* **167**, 3555–3558.
16. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Nunez, G. (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J. Biol. Chem.* **276**, 4812–4818.
17. Kaisho, T., Takeuchi, O., Kawai, T., Hoshino, K., and Akira, S. (2001) Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J. Immunol.* **166**, 5688–5694.
18. Henneke, P. and Golenbock, D. T. (2001) TIRAP: how Toll receptors fraternize. *Nat. Immunol.* **2**, 828–830.

Historical Perspectives

**Saburo Yamamoto, Toshiko Yamamoto,
and Tohru Tokunaga**

1. INTRODUCTION

The concept of immunostimulatory DNA sequence was borne in a long series of studies on BCG-mediated tumor resistance. DNA purified from BCG inhibited the growth of various guinea pig and mouse tumors, and augmented natural killer (NK) cell activity and induced interferons (IFNs) from mouse spleen cells (1,2). Further, we found two remarkable facts that

1. DNAs from bacteria, but not animals and plants, showed the above-mentioned immunological activity (3).
2. The activity was completely dependent on particular base sequences having CpG motifs (4).

Research interests of immunostimulatory DNA sequences were galvanized in 1995 by the report of Krieg showing murine B-cell activation with bacterial DNA containing CpG motifs (5). Within a short period of time, a huge number of papers have been published in this field, and the study has expanded rapidly and extensively. Now, it includes a number of research fields, for example, host-defense mechanisms against infection, allergy, autoimmune diseases, cytokine networks, plasmid vaccination, and therapeutic application of certain diseases (6–7,9–11). The response of higher animals against immunostimulatory DNA must be the most primitive but important mechanism for self-nonsel self discrimination against foreign DNA.

2. THE DISCOVERY OF ANTITUMOR ACTIVITY OF DNA FROM BCG

Trials of cancer immunotherapy with BCG were carried out on a world-wide scale in the 1970s and contributed much information to the fields of basic and clinical immunology. During this decade, efforts were made to isolate components of BCG possessing antitumor activity and diminished

adverse effects. While trying to obtain water-soluble components of BCG, we found that BCG cytoplasm precipitated by streptomycin sulfate contained substances strongly active against guinea pig hepatoma (1).

Streptomycin sulfate precipitate of BCG was a complex of various components—protein, nucleic acid, lipid, and sugar. Repeated injections of this fraction into guinea pigs caused severe anaphylactic shock. To avoid this phenomenon, we further extracted the fraction of streptomycin sulfate precipitate with hot water and found that the heat extract kept a strong ability to inhibit tumor growth and did not cause any adverse effect. This fraction was further purified with multi-step procedures, and finally a fraction designated MY-1 was obtained.

MY-1 composed of 98% nucleic acid (70% DNA, 28.0% RNA) and its protein and sugar content were only 1.3 and 0.2%, respectively. The DNA contained in MY-1 was single-stranded as judged by the results of an ultracentrifuge analysis, a hydroxy apatite column chromatography, and a measurement of temperature-absorbance.

MY-1 showed stronger antitumor activity than the streptomycin sulfate precipitate. No macroscopic inflammatory change was observed at the injection sites of MY-1, although a typical delayed-type inflammatory reaction was seen at the site of BCG injection. DNA contained in MY-1 was essential for the antitumor activity because the fraction of MY-1 digested with RNase showed higher antitumor activity than MY-1, although MY-1 after digestion with DNase that contained mostly RNA had reduced activity. Until recently, DNA had only been considered as the blueprint of life and was thought to be immunologically uniform and essentially inert. MY-1 is unique because its component is mostly nucleic acid and its activity is ascribed to DNA.

3. OLIGODNA SEQUENCES CONTAINED IN MY-1

A gel filtration column chromatography indicated that MY-1 was distributed over a broad range of molecular size, and its elution peak corresponded to 45 bases. To determine whether the immunostimulatory activity of MY-1 was dependent on base sequence, 13 different 45-mer single-stranded oligoDNAs were synthesized and evaluated their immunostimulatory activity to augment natural killer (NK) cell of normal mouse spleen cells (12). Six of the 13 oligoDNAs, i.e., A3, A4, A6, A7, M3, and alpha-1, showed strong activity, whereas the others did not. Two oligoDNAs, A4 and A2, were selected as the representative of active and inactive oligoDNA, respectively. The cytotoxicity of the spleen cells was elevated remarkably by A4 in a dose-dependent manner, although the cells incubated with A2 showed

no significant change in the activity at any concentration. The palindromic sequence (5'-GACGTC-3') was replaced with each of the 63 theoretically possible 6-mer palindromes in the sequence of A4a, 5'-accgatGACGTCgcc ggtgacggcaccacg-3', and the resulting A4a analog were assayed for the ability to enhance NK cell activity (13). Only 8 oligoDNAs including one of the following palindromic sequences: AACGTT, AGCGCT, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC, and TCGCGA, showed the stronger activity than that of A4a. All the potent palindromes included one or more 5'-CpG-3' motif(s).

4. IMMUNOSTIMULATORY ACTIVITY OF THE DNA PREPARED FROM VARIOUS SOURCES

The DNA-rich fraction from six species of bacteria, namely *Streptomyces aureofaciens*, *Mycobacterium bovis* BCG, *Pseudomonas putida*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*, exhibited the immunostimulatory activity similar to MY-1, but DNA from calf thymus and salmon testis did not (3). In addition to these eight DNA fractions tested, 23 kinds of DNA samples, all of which were extracted from various sources by the Marmur's method, were examined for augmentation of NK cells and induction of IFN in vitro. Each of the DNA samples from *Mycrococcus lysodeikticus*, *Mycobacterium bovis* BCG, *Escherichia coli*, and *Mycoplasma pneumoniae* strongly augmented NK activity and induced interferon (IFN). Biological activities of the DNA sample from *Clostridium perfringens* were relatively low, but were statistically significant. The DNA sample from X 174 phage showed strong activities, and that from adenovirus exhibited less but significant activities. In the DNA samples of the four species of invertebrate, the sample from silkworm showed strong activities, and those from sea urchin, lobster, and mussel showed less but significant activities. In contrast, all of the DNA samples from 10 different species of vertebrate, including five of mammal, and from two species of plant exhibited no activity (Fig. 1).

The activity of the bacterial DNA fractions were not influenced by the presence of polymixin B, an inhibitor of lipopolysaccharide (LPS), and were observed even in the spleen cells from LPS-insensitive C3H/HeJ mice, indicating that the activity could not be attributable to possibly contaminating LPS. The profiles of agarose-gel electrophoresis were essentially the same in all of the DNA fractions. UV absorbance at 260nm of MY-1 and the DNA from calf thymus decreased to the same proportion by DNase treatment. The (G + C) content of the bacterial DNAs used varied from more than 70% to less than 30%, all of which were active, and those of calf and salmon DNA

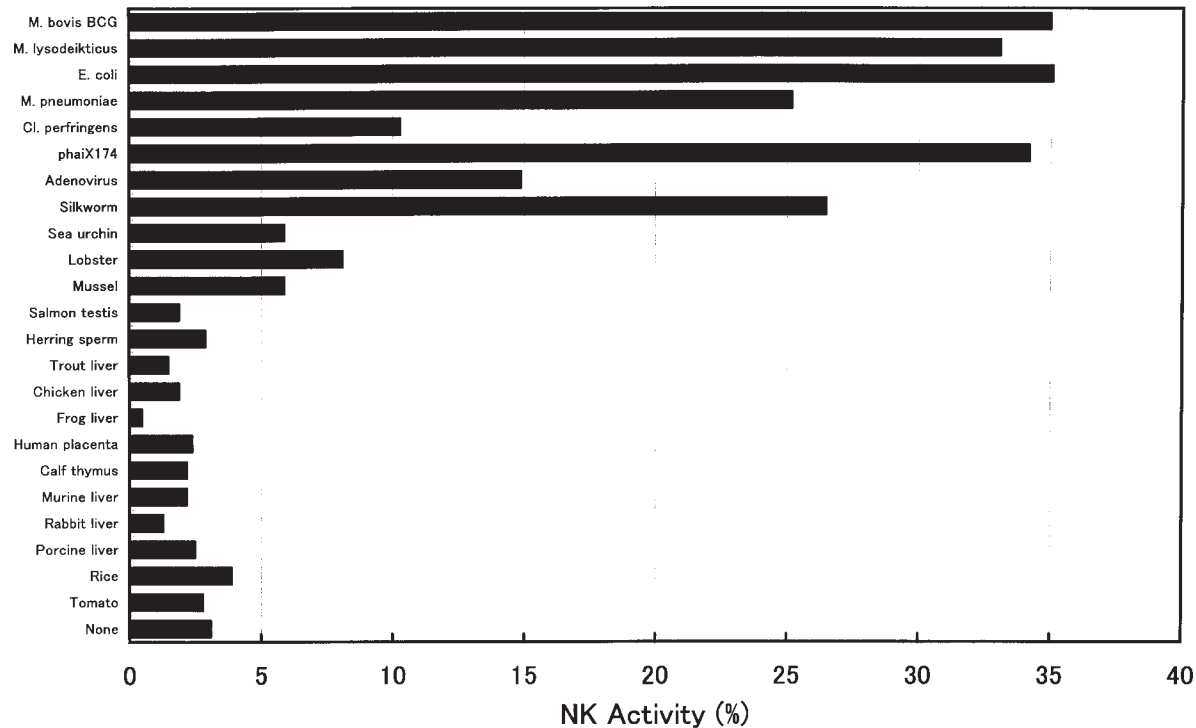


Fig. 1. Augmentation of NK activity by the DNA sample from various sources. BALB/c mouse spleen cells ($1 \times 10^7/\text{mL}$) were incubated with $10 \mu\text{g}/\text{mL}$ of each of the DNA samples for 20 h, and centrifuged. The cell fractions were assayed for NK activity to measure by a 4 h ^{51}Cr -release assay against YAC-1 lymphoma cells as target cells. M.bovis: *Mycobacterium bovis*; M. lysodeikticus: *Micrococcus lysodeikticus*; E. coli: *Escherichia coli*; M. pneumoniae: *Mycoplasma pneumoniae*; Cl. perfringens: *Clostridium perfringens*.

were 50.2% and 40.2%, respectively. No correlation between (G + C) content and immunostimulatory activity of DNA was found. Methylation of the cytosine of AACGTT resulted in a significant decrease of its activity (14). We also found that the incubation of *Escherichia coli* DNA with CpG methylase reduced the IFN-inducing activity with a lapse of incubation time. We surveyed the incidence frequency of the nine potent palindromic sequences in some of the cDNA sequences in the GenBank DNA Data Base; we chose one or more sequences at will from the cDNAs of 17 species. The summed incidences of the potent palindromic sequences in all of the cDNA sequences from vertebrates and plants were less than 1.0 in 1000 base-pairs, whereas those from most of the bacterial, viruses, and silkworm were larger than 1.0. There were some exceptions; the incidence, for instance, in the cDNA sequences from *Mycoplasma pneumoniae* and *Clostridium perfringens* was very low (0.4–0.2), but their activities were high. These discrepancies may be due to our limited analysis of only the tiny parts of the huge genomic DNA. The incidence of particular 8-, 10- or 12-mer palindromic sequences, which show stronger immunostimulatory activity than particular hexamer (14), was not taken into account, either. Bird described that CpG in bulk vertebrate DNA occurs at about one-fifth of the expected frequency (15). We think that the different frequency of potent sequences in DNA between vertebrate and invertebrate DNA must be another reason for the difference in activities.

REFERENCES

1. Tokunaga, T., Yamamoto, H., Shimada, S., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physico-chemical characterization and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–962.
2. Shimada, S., Yano, O., Inoue, H., et al. (1985) Antitumor Activity of the DNA Fraction from *Mycobacterium bovis* BCG. II. Effects on Various Syngeneic Mouse Tumors. *J. Natl. Cancer Inst.* **74**, 681–688.
3. Yamamoto, S., Yamamoto, T., Shimada, S., et al. (1992) DNA from Bacteria, but Not from Vertebrates, Induces Interferons, Activates Natural Killer Cells and Inhibits Tumor Growth. *Microbiol Immunol.* **36**, 983–997.
4. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique Palindromic Sequences in Synthetic Oligonucleotides are required to Induce IFN and Augment IFN-Mediated Natural Killer Activity. *J. Immunol.* **148**, 4072–4076.
5. Krieg, A. M., Yi, A.-K., Matson, S., et al. Koretzky, G. A. and Klinman, D. M. (1995) CpG motifs in bacterial DNA trigger direct B cell activation. *Nature* **374**, 546–549.

6. Goodman, J. S., Van Uden, J. H., Kobayashi, H., Broide, D. and Raz, E. (1998) DNA immunotherapeutics: New potential treatment modalities for allergic disease. *Int. Arch. Allergy Immunol.* **116**, 177–187.
7. Krieg, A. M., Love-Homan, L., Yi, A.-K. and Harty, J. T. (1998) CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* **161**, 2428–2434.
8. Lowrie, D. B., Tascon, R. E., Bonato, V. L. D., et al. (1999) Therapy of tuberculosis in mice by DNA vaccination. *Nature* **400**, 269–271.
9. Pisetsky, D. S. (1996) Immune Activation by Bacterial DNA: A New Genetic Code. *Immunity* **5**, 303–310.
10. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
11. Zimmermann, S., Egeter, O., Hausmann, S., et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 response in lethal murine leishmaniasis. *J. Immunol.* **160**, 3627–3630.
12. Tokunaga, T., Yano, O., Kuramoto, E., et al. (1992) Synthetic Oligonucleotides with Particular Base Sequences from the cDNA Encoding Proteins of *Mycobacterium bovis* BCG Induce Interferons and Activate Natural Killer Cells. *Microbiol. Immunol.* **36**, 55–66.
13. Kuramoto, E., Yano, O., Kimura, Y., et al. (1992) Oligonucleotide sequence required for natural killer cell activation. *Jpn. J. Cancer Res.* **83**, 1128–1131.
14. Sonehara, K., Saito, H., Kuramoto, E., Yamamoto, S., Yamamoto, T., and Tokunaga, T. (1996) Hexamer palindromic oligonucleotides with 5'-CG-3' motif(s) induce production of interferon. *J. IFN Cytokine Res.* **16**, 799–803.
15. Bird, A. P. (1986) CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209–213.

PART II

RECEPTORS AND SIGNALING

Signal Transduction Pathways Activated By CpG-DNA

Hans Häcker

1. INTRODUCTION

Since the initial observation that bacterial DNA is recognized by and activates cells of the immune system (reviewed in 1), substantial progress has been made with respect to the understanding of the molecular mechanisms involved. This bears upon both sides, the immunostimulatory DNA as a ligand and the immune cell with its receptor and signaling systems. In the meantime, it has been well established that the stimulatory capacity of bacterial DNA depends on short sequences with a central, unmethylated CG, called the CpG-motif (2,3). This stimulatory information can be transferred to single-stranded oligonucleotides (ODN). So far as it is known, all stimulatory activities of bacterial DNA are reflected in such ODNs. Therefore, these single-stranded ODNs might be regarded as the active principle of immunostimulatory DNA. It is however important to note that—as worked out with sophisticated arrays of ODNs—there is a clear species specificity in respect to the sequences active in mouse vs humans. Hence, it might be speculated that double-stranded bacterial DNA represents a pool of various stimulatory sequences that are recognized by specific but species-dependent receptor systems. DNA that harbors immunostimulatory capacity owing to CpG-motifs is collectively referred to as CpG-DNA.

The cell types that respond to CpG-DNA are primarily cells of the innate immune system, like dendritic cells (DCs) and macrophages as well as B-cells (reviewed in 4). Although great headway has been made in respect to the molecules involved in recognition and signal transduction of CpG-DNA, this must be considered in the context of two other, major observations of the last years: The definition of the “Toll-like receptors (TLR)” as molecules used by immune cells to recognize invariant pathogen derived mol-

ecules and the characterization of the I κ B Kinase complex (IKK) as a central regulator of the NF- κ B pathway triggered by inflammatory stimuli.

In this chapter, the cellular molecules that have been found to be critically involved in recognition and signal transduction of CpG-DNA will be discussed. As these molecules have primarily been characterized as components of the TLR-, the NF- κ B, and the Mitogen-activated protein kinase (MAPK)-pathways, a brief summary of these pathways is provided.

2. THE TOLL-LIKE RECEPTOR PATHWAY

Cells of the innate immune response like DCs and macrophages are able to recognize and to respond to a wide variety of different pathogen-derived products. These include constituents of the bacterial cell wall of gramnegative and grampositive bacteria such as lipopolysaccharides (LPS) and peptidoglycans (PGN), respectively. Because of this apparently promiscuous feature, for a long time, this part of the immune response has been considered to be nonspecific. Only a few years ago, it became clear that this phenotype is owing to the expression of a repertoire of particular receptors of different specificity, the TLRs. Obviously, individual receptors recognize certain, conserved constituents of pathogens. Because these constituents recognized by TLRs typically belong to a group of chemically similar molecules, for example various forms of LPS, rather than represent one particular molecule, these ligands have been termed pathogen associated molecular patterns (PAMPs). In respect to their specificity, two out of ten known mammalian TLRs have been especially well characterized: TLR2, which mediates responsiveness to PGN and bacterial lipoproteins (BLP) and TLR4, which recognizes LPS and lipoteichoic acids (5). Although direct binding to the receptors has not been demonstrated unequivocally (for discussion see 6), the extracellular domain of the receptor seems to be involved in recognition of the ligands. Consecutively, oligomerization of TLR-chains appears to initiate signal transduction via the intracellular part of the receptor (Fig. 1). Interestingly, the intracellular part of TLRs shares strong homology to receptors of the IL-1 family, the IL-1 receptor (IL-1R) and IL-18 receptor. Due to this homology, this part of the receptor is referred to as TLR/IL-1R (TIR)-homology domain. Signaling via the IL-1R complex has been partially characterized. Receptor engagement leads to recruitment of the adapter molecule MyD88 that directly binds to the TLR/IL-1R-complex (7). Members of the IRAK family (IRAK-1,-2,-m) and the adapter molecule TRAF6 are recruited to this complex. The exact molecular processes that lead to recruitment of TRAF6 and the role of the IRAKs has been defined only partially. However, TRAF6 has been cloned as a molecule that directly

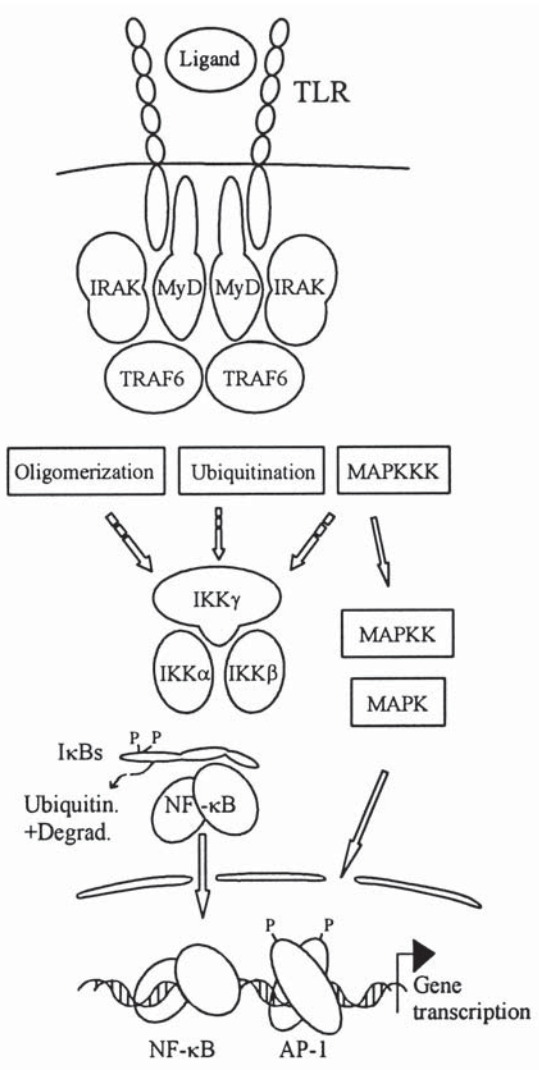


Fig. 1. Model of the Toll-like receptor pathway. MyD = MyD88, myeloid differentiation marker 88; IRAK, IL-1R-associated kinase; IKK, IκB kinase; TRAF6, tumor-necrosis-factor receptor-associated factor 6.

binds to CD40 (8). Moreover, it is known that isolated oligomerization of the effector domain of TRAF6 (mimicking CD40-dependent TRAF6-oligomerization) is sufficient to induce downstream signaling (9). Taken together, these data suggest that oligomerization of TIR-domains of TLR/IL-1R chains initiate recruitment and oligomerization of MyD88 and TRAF6, set-

ting up the signaling platform for the activation of downstream kinases. Among these kinases, some have been found to be activated by CpG-DNA and to regulate important effector functions. These include kinases of the IKK-complex, the mitogen activated protein kinases (MAPKs), Jun N-terminal kinases (JNK) 1/2, p38 kinases, and the extracellular-signal-regulated kinases, (ERK) 1/2.

3. THE NF- κ B ACTIVATION PATHWAY

The transcription factor NF- κ B is composed of dimers of the Rel family of DNA-binding proteins which include RelA/p65, p50, p52, c-Rel, and RelB (for review see 10). Expressed in virtually all cell types of eukaryotes, a hallmark of NF- κ B is its tight regulation and inducibility. In the immune system activation of NF- κ B in general, leads to an enhancement of immune responses. These include expression of soluble factors like cytokines and chemokines, membrane proteins, involved in costimulation and adhesion and proteins regulating cell survival. Activation of NF- κ B is controlled by inhibitory proteins, the I κ Bs. I κ B is also a family of molecules that includes I κ B α , I κ B β , I κ B γ , I κ B ϵ , and Bcl3. These inhibitory molecules bind preformed NF- κ B dimers thereby inhibiting nuclear translocation and DNA-binding of the transcription factor. The best characterized members of the I κ B family are I κ B α and I κ B β . Cell stimulation, for example by proinflammatory cytokines like TNF or IL-1 leads to phosphorylation of two conserved Serin residues within the N-terminal regulatory domain, followed by rapid ubiquitination and degradation of the I κ Bs by the 26S proteasome. Subsequently, the released NF- κ B dimer translocates to the nucleus, free to bind NF- κ B enhancers and to activate gene transcription. A kinase complex, the I κ B kinase complex (IKK) that phosphorylates I κ Bs at the critical Serin residues upon stimulation by proinflammatory stimuli has been characterized (for review see 11). This high molecular weight complex of 700–900 kD is composed of three proteins, now named IKK α , IKK β , and IKK γ . Two of these proteins, IKK α and IKK β have catalytic activity whereas IKK γ seems to be required for the formation of the complex, and the transduction of upstream signals toward the activation of the catalytic subunits IKK α and IKK β . Although the different IKK constituents seem to be represented in equal stoichiometric ratios in the cell types investigated so far, gene targeting experiments generating cells that lack the one or the other IKK-protein show that they have nonredundant functions. Although IKK γ seems to be required for all stimuli leading to activation of IKKs (12,13), IKK β is necessary for signaling by proinflammatory signals like IL-1, TNF, and LPS (14,15). IKK α however seems to be mainly required for cell differentiation of keratinocytes (16).

Although the molecular and functional properties of the IKK complex have been carefully studied, it is still a largely unanswered question, how the signal emerging from activated receptor complexes with oligomerized adapter molecules like the TRAF proteins is transduced to activate the IKK complex. Some kinases like NIK (NF- κ B inducing kinase) or MEKK-1 (*see* Section 4) have been implicated as upstream kinases, largely based on experiments with overexpressed proteins or dominant negative mutants (for discussion *see* 17). Experiments employing knockout cells indicate that these molecules might be involved in NF- κ B activation through specific stimuli, like NIK in the lymphotoxin- β receptor pathway, however, they seem to play no significant role for major IKK-activation pathways like the TNF receptor- or TLR/IL-1R-pathway (18). A very recent report implicates TRAF6 as a ubiquitin ligase. Using sophisticated in vitro reconstitution assays, Deng et al. propose that ubiquitination of a so far not defined protein is a prerequisite for IKK activation (19). Another intriguing possibility has been put forward by Inohara et al. and Poyet et al. (20,21). Using fusion proteins with domains for artificial oligomerization they provide evidence that enforced proximity, induced by oligomerization of particular molecules, i.e., Nod-1, Rick, and RIP, and oligomerization of IKK-subunits is sufficient to trigger IKK activation. In its extreme interpretation, this could mean that all molecules required to close the gap between receptor and IKK have already been defined, and it is solely its molecular cooperation that must be characterized. Certainly, none of the described possibilities are exclusive of the other.

3.1. CpG-DNA Induces NF- κ B Activation through the I κ B Kinase Complex

The first data indicating that CpG-DNA activates NF- κ B were obtained in experiments with murine macrophages. Using plasmid DNA (as a source of bacterial DNA) and synthetic ODNs containing palindromic CpG-motifs as stimuli, Stacey et al. demonstrated enhanced NF- κ B binding activity as well as NF- κ B-dependent gene transcription in macrophages upon stimulation with these agents (22). This activity strictly depended on the presence of unmethylated CpG-motifs. Later on, Yi et al. showed that stimulation of WEHI-231 B-cells with CpG-ODN induced degradation of I κ B α and I κ B β , leading to nuclear translocation of p50/c-Rel (23), p50/p65 and p50 homodimers (24). Concurrent with activation of NF- κ B, production of reactive oxygen species (ROS) has been demonstrated in these cells (23). Additionally, treatment with pyrrolidine dithiocarbamate (PDTC) or N-acetyl-L-cysteine (NAC) suppressed TNF- and IL-6-secretion from CpG-DNA-stimulated cells (23,25), implying an important role for the redox state

of the responding cell during stimulation. Notably, almost all inducers of NF- κ B tested so far can be blocked by antioxidants (26–28). This could mean that production of ROS is an important and general way to activate NF- κ B, however, for none of the stimuli tested, the critical step during signaling which depended on ROS generation could be demonstrated so far. Although there is no doubt about the principal importance of the redox state of cells, it is still not clear whether its altered dynamics during cell stimulation is important for NF- κ B activation.

Degradation of I κ B α and I κ B β and specific phosphorylation of I κ B α at Ser32 and Ser36 strongly suggested that the IKK complex would be activated by CpG-DNA. Indeed, using HA-tagged IKK α or antibodies against IKK α to precipitate the IKK complex, followed by in vitro kinase assays, it could directly be demonstrated that this complex is activated by CpG-DNA (29,30). Using IKK β -deficient cells it was demonstrated that for CpG-DNA—as for other proinflammatory stimuli like LPS—the IKK β subunit is critical for the catalytic activity of the IKK complex. Moreover, CpG-DNA-induced NF- κ B translocation was completely abolished in IKK β -deficient macrophages (30).

Taken together, CpG-DNA activates NF- κ B in all responsive cell types investigated so far. Activation is accomplished by the classical way, i.e., activation of the IKK complex which phosphorylates critical Serine residues in the I κ Bs, followed by rapid ubiquitination and degradation of these proteins and subsequent nuclear translocation of the freed NF- κ B dimers.

3.2. Activation of NF- κ B is Essential for CpG-DNA-Induced Effector Functions

The outstanding role of NF- κ B during regulation of immune responses has been known for a long time, and many genes involved, like IL-12, IL-6, and IL-1, contain characterized NF- κ B binding sites in their promoter regions (reviewed in 10). Because of the central role of the IKK complex for activation of NF- κ B by almost all stimuli investigated so far, the use of cells from IKK-deficient mice is of particular interest to define what role NF- κ B plays for the regulation of distinct genes, activated by different stimuli like CpG-DNA. Two genes, IL-12 p40 and IL-6, have been directly investigated in cells from IKK β -deficient cells (30). Whereas both genes contain NF- κ B binding sites in their promoter regions, IL-12 is particularly interesting, as this cytokine plays a pivotal role for the regulation of adaptive immune responses by innate immune cells. Two transcription factors, NF- κ B and CEB/P, seem to be important for their inducibility by PAMPs like LPS (31,32). The NF- κ B site has been shown to bind specifically p50/cRel dimers which are critical for IL-12 p40 induction, whereas IL-6 requires primarily p50/p65 dimers for gene activation (33). According to the limiting role of

IKK for CpG-DNA-induced NF- κ B activation, macrophages from IKK β -deficient (and additionally TNFRp55-deficient) mice have severe defects in IL-12 and IL-6 production in response to CpG-DNA (30).

Although IL-12 and IL-6 are the only CpG-DNA-induced genes investigated so far, other effector functions of CpG-DNA like its antiapoptotic effect in B cells or its role for regulation of costimulatory molecules await further characterization in respect to their dependency on IKK and NF- κ B activation.

4. MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS

Mitogen-activated protein kinases (MAPKs) represent a group of serine/threonine-specific kinases that have been found to be activated in response to a variety of stimuli (reviewed in 34). MAPKs, which are involved in the regulation of certain transcription factors and other regulatory proteins, are themselves activated by upstream kinases called MAPK kinases or extracellular-signal-regulated kinase (ERK) kinases (MAPKKs/MEKs) and MAPKK kinases/MEK kinases (MAPKKKs/MEKKs). In vertebrates, at least three MAPK pathways can be distinguished. Signals from different sources, for example membrane receptors, are sensed and integrated to transduce them into specific gene expression. In general, the different MAPK pathways seem to be organized as sequentially operating modules which are held together by partially characterized scaffold proteins forming complexes with kinases belonging to a given cascade. This organization seems to contribute to the high degree of specificity (35,36). Named after the last MAPK in the corresponding cascade, they are referred to as the extracellular-signal-regulated ERK/MAPK pathway, the p38 MAPK pathway, and the c-Jun *N*-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway. Members of the JNK- and p38-groups of kinases are also commonly referred to as stress kinases.

The ERK/MAPK pathway has originally been found to be activated by growth factors and phorbol esters. The importance of the ERK pathway for mitogenicity of growth factors and oncogenic transformation of cells through constitutive activity of members of this pathway has been established (37). However, dependent on the cell type, other stimuli, for example proinflammatory cytokines or PAMPs, strongly trigger the ERK pathway (*see* Section 4.2.).

The p38 and JNK MAPKs are activated by different stimuli, including proinflammatory cytokines such as TNF and IL-1, stress signals, ultraviolet (UV) light and PAMPs like LPS. (38–42).

4.1. CpG-DNA Activates Stress Kinases and the Transcription Factor Activating Protein 1 (AP-1)

The first hint that CpG-DNA might activate cells via signal transduction pathways used by other classical receptor ligands came from transfection experiments utilizing AP-1 luciferase reporter plasmids (43). Stimulation of

ANA-1 macrophages with plasmid DNA or CpG-ODN led to significant AP-1-driven transcriptional activity. This effect was strictly dependent on unmethylated CpG-motifs.

The transcription factor AP-1 is composed by members of the Jun, Fos, and ATF (activating factor) families, which form homodimers and heterodimers. With respect to participation in AP-1 complexes, c-Jun, c-Fos, and ATF-2 are especially well characterized. The transcriptional activity of AP-1 is regulated at different levels (44). Both the abundance of the AP-1 protein complex and its transcriptional activity are regulated by MAPK pathways. C-Fos is mainly regulated at the transcriptional level whereas c-Jun is regulated both at the transcriptional level and by specific phosphorylation at two sites, Ser63 and Ser73, which enhance the transactivating potency of the AP-1 complex (45). The kinases that phosphorylate c-Jun at these sites are the JNKs (46). ATF-2, which is constitutively expressed, is regulated by phosphorylation by p38 and JNK (47,48).

As mentioned previously, CpG-DNA induces transcriptional activity of AP-1 in ANA-1 macrophages. Gel shift assays in these cells revealed basal AP-1-binding activity but only a slight increase during the first 4 h of stimulation (43). However, c-Jun, which was contained in the AP-1 complex, was found to be phosphorylated at Ser73 and Ser63 within 10 min and remained in this state over the next 4 h. With comparable kinetics, ATF2 was found to be phosphorylated at sites critical for its transactivation potency. Because of the correlation of the transcriptional activity and phosphorylation of c-Jun it might be surmised that in these cells AP-1 is primarily regulated by phosphorylation of c-Jun. In contrast to ANA-1 macrophages, stimulation of WEHI-231 B-cells with CpG-DNA leads to phosphorylation of c-Jun and concomitant increased AP-1 binding activity (49). Obviously, different cell types regulate this complex transcription factor by different means.

The kinases that regulate transcriptional activity of AP-1 by phosphorylation of c-Jun and ATF2 are the JNK1/2 and p38 kinases. Indeed, in different cell types activation of JNK1/2 and p38 has been demonstrated as well as phosphorylation of MKK4, one of the upstream kinases of JNK1/2. Notably, JNK1/2 and p38 have been found to be activated in all cells that are responsive to CpG-DNA, i.e., macrophages, DCs, and B cells. Therefore, activation of these stress kinase pathways seems to be a general phenomenon of CpG-DNA-induced cell activation.

4.2. CpG-DNA ACTIVATES THE ERK/MAPK PATHWAY IN A CELL-TYPE SPECIFIC WAY

It has been established for a long time that the ERK/MAPK pathway is activated by growth factors. However, dependent on the cell type, other

stimuli like proinflammatory cytokines and LPS can also activate this pathway (50). Using phospho- (activation-) specific antibodies against ERK1/2 and in vitro kinase assays it has been demonstrated that CpG-DNA leads to significant activation of this pathway in macrophage cell lines, as well as primary—peritoneal and bone marrow derived—macrophages (51,52). In these cells the kinetics of ERK1/2 activity resembles that of IKK-, JNK-, and p38, implying that these pathways are not triggered in a sequential way dependent on each other, but rather utilize a common upstream molecule. By means of phosphospecific antibodies against MEK1/2, upstream kinases of ERK1/2, and transfection experiments with dominant negative MEK1, MEK could be defined as the upstream kinase of ERK in CpG-DNA-stimulated cells (51). Still, the upstream kinase of MEK, which is activated by CpG-DNA is not known. Although the kinase RAF and the GTPase RAS are critically involved in activation of MEK by growth factors, for example epidermal growth factor, in the case of LPS (which seems to be the most closely related stimulus to CpG-DNA investigated so far) RAS-dependent and-independent pathways seem to exist (53,54).

Notably, activation of ERK by CpG-DNA appears to be strictly cell type specific. Although macrophages strongly activate ERK in response to CpG-DNA, neither DCs nor B-cells seem to activate this pathway (51). This pattern of kinase activation has important implications for cytokine production by macrophages and DCs (*see* Section 4.3.).

4.3. CpG-DNA-Induced MAPK Pathways Regulate Effector Functions of Immune Cells

Although a huge number of effector functions are triggered by CpG-DNA, the role of MAPK pathways for these functions has been only partially elucidated. Indeed, until now no effector function has been linked to JNK-activation by CpG-DNA although this pathway is strongly activated in all cell types found to be responsive to CpG-DNA. This is primarily owing to different practical reasons: first, there is no specific inhibitor of JNKs available, second, JNK1 and JNK2 seem to have redundant roles, which complicates the work with knock-out animals and third, JNK1/2 can be activated by more than one upstream kinase, MKK4 and MKK7, where MKK4-deficient mice are not viable. Together, evaluation of this pathway for CpG-DNA-dependent effector functions awaits further development of basic tools.

In contrast to JNK, a role for p38 could be defined for different cytokines like IL-12, TNF, and IL-6. These cytokines, produced by DCs or macrophages seem to be positively regulated by p38 (43,55). This interpretation is mainly based on experiments using a specific p38 inhibitor, but has been substantiated in respect to IL-12 by MKK3-deficient cells. Stimulation of

these cells with LPS leads to reduced p38-activation and impaired IL-12 production (56). Using p38 inhibitors and dominant negative mutants of p38, MKK3, and MKK6, p38 has been concluded to act at the promoter level. In contrast, IL-6 seems to be regulated by p38 by increasing its mRNA stability (57) while TNF production has been shown to be controlled on a translational level (58). Although the data known so far indicate that p38 is involved in regulation of a panel of inflammatory cytokines, it is important to note that the molecular downstream targets of p38 activation, which impinge directly on the respective effector function, are not known for one of them.

Also, the functional significance of the ERK/MAPK pathway for stimulation by CpG-DNA is only partially defined. However, the fact that ERK1/2 is activated in a cell type specific manner led to an interesting observation in respect to the role of these kinases for regulation of IL-12 and TNF. Although TNF is positively regulated by ERKs, an effect that has already been described for LPS-stimulated cells, ERK activation obviously suppresses IL-12 production, an effect that can be traced back to the activity of the IL-12 p40 promoter. Accordingly, macrophages that activate ERK in response to CpG-DNA (or LPS [59]) produce only small amounts of IL-12. In contrast, DCs that do not activate ERK, produce more than two orders of magnitude more IL-12 than macrophages (51). Whereas this example shows how complex effector functions are regulated by the balance of positive and negative signals, it also elucidates how cells of various differentiation status accomplish their cell type specific effector repertoire.

5. CpG-DNA ACTIVATES CELLS VIA THE TLR-PATHWAY

Under Subheading 2., the Toll-like receptor signaling pathway has been described. There were several reasons to investigate this pathway in respect to CpG-DNA:

First, CpG-DNA displays the typical characteristics of PAMPs, pathogen associated ligands that have been shown to signal via TLRs (60): immunostimulatory DNA (CpG-DNA) is structurally different from the host DNA of mammals, it is shared among wide groups of pathogens, allowing one (ore a few) receptor(s) to recognize larger groups of microbes, and it is essential for the survival of the microbial organisms, preventing escape mutants. Second, with respect to many functional aspects of the cellular response like cytokine production and signaling events, CpG-DNA resembles other PAMPs like LPS. Third, CpG-DNA signaling possesses important functional parallels to signaling by CD40, a transmembrane receptor that is required for T cell dependent activation of antigen presenting cells (APC). CD40 engagement evokes effector functions such as

upregulation of costimulatory molecules and production of IL-12, key marks of CpG-DNA-triggered activation of APC (61).

5.1. CpG-DNA Activates Immune Cells via MyD88 and TRAF6

As detailed in Subheading 2, the molecules MyD88 and TRAF6 are crucial components of the TLR/IL-1R-pathway. As a first step in the investigation of the involvement of TRAF6 and MyD88 in CpG-DNA-signaling, dominant negative mutants of these proteins were transfected in RAW264.7 macrophages. And indeed, both mutants specifically inhibited activation of IKK, JNK and—as a consequence—induction of the IL-12 p40 promoter (29). Consistent with these results, MyD88-deficient cells were found to be nonresponsive to CpG-DNA in all functions tested so far: B-cell proliferation, production of IL-12, TNF, and IL-6 and degradation of I κ B α (29,62). Taken together, these data showed that CpG-DNA signals via the TLR-pathway, engaging MyD88 and TRAF6 to activate downstream kinases like IKK and JNK and thereby induce effector functions such as cytokine production and cell proliferation.

5.2. CpG-DNA Activates Immune Cells via Toll-like Receptor 9

MyD88 is an adapter molecule that has been found to bind directly to the intracellular part of TLR/IL-1R family members through its C-terminal TIR-domain by homophilic interaction. Therefore, on the basis of the data described previously, it has been compelling to postulate a TLR family member as a critical part of CpG-DNA-dependent cell activation. And indeed, very recently a TLR has been described that seems to have the characteristics of the CpG-DNA-receptor postulated. This receptor, named TLR9, has been genetically defined by different groups in parallel as a typical member of the TLR family, containing leucine-rich repeats (LRRs) at its N-terminal side and a TIR-domain at its C-terminal end (63,64). Two hydrophobic stretches, one at the very N-terminus and one between the LRRs and the TIR-domain, could be interpreted as endoplasmatic leader signal and trans-membrane region, respectively. Therefore it might be suspected that the orientation of TLR9 resembles other TLRs with the extracellular LRR region and the intracellular TIR domain. mRNA of TLR9 was found to be selectively expressed in immune cell rich tissues like spleen (64). Hemmi et al. investigated its functional role by generation of TLR9-deficient mice (65). Using a panel of in vitro and in vivo assays, the authors demonstrated that cells from these mice were no longer responsive to CpG-ODN, but exhibited normal responses to other PAMPs like LPS and PGN. In vitro, these assays included B-cell proliferation, cytokine production from macrophages (TNF, IL-6, IL-12 p40), upregulation of costimulatory molecules from DCs

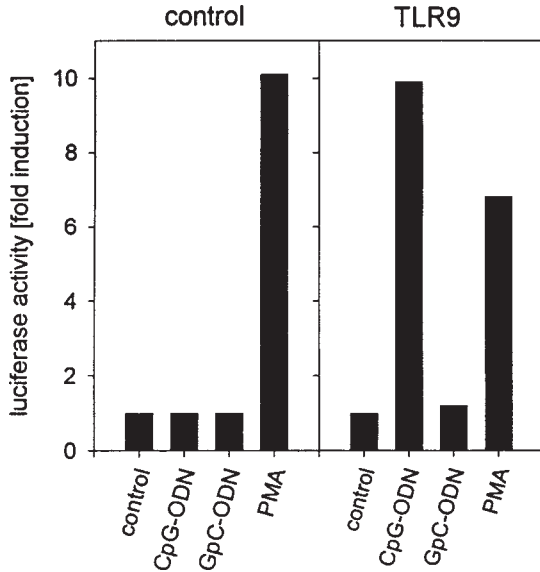


Fig. 2. TLR9 confers responsiveness to CpG-DNA. Human embryonic kidney cells (HEK 293T) were transiently transfected with an NF- κ B-dependent luciferase reporter vector and an expression vector for murine TLR9 (a kind gift from Dr. S. Bauer) as indicated. After transfection, cells were left untreated or stimulated with CpG-ODN, GpC-ODN (1 μ M each, sequences as in 43) or phorbol ester (PMA, 10 ng/mL) for 8 h and luciferase activity was determined.

and activation of NF- κ B, JNK1/2 and IRAK-1 in macrophages. In vivo, TLR9-deficient mice failed to produce cytokines in response to CpG-DNA (TNF, IL-12 p40, IL6) and were resistant against injection of CpG-ODN in the lethal shock model of D-galactosamine-sensitized mice (65). Furthermore, cells that are naturally not responsive to CpG-DNA, for example HEK293T cells, can be converted to CpG-DNA-responsive cells by sole expression of TLR9 cDNA (unpublished data, Fig. 2). These cells gain the functional characteristics of naturally responsive cells in that they can be activated by bacterial DNA and CpG-ODN in a strictly CpG-dependent manner: CG-specific methylation or inversion of the central CG abolishes their capacity to activate TLR9-expressing cells. As in innate immune cells, TLR9-dependent activation of NF- κ B is MyD88-dependent (unpublished observation). Taken together, all pieces of information seem to be complete to rate TLR9 as the critical signaling receptor in CpG-DNA-dependent cell activation. One piece of information that is still missing is whether TLR9 is the CpG-receptor itself, directly binding to and recogniz-

ing the CG-specific sequence information contained in stimulatory DNA. One important step in this direction will be the use of gain of function assays as mentioned previously, using murine and human TLR9-transfectants in comparison to see whether the species-specificity of certain CpG-ODN-sequences are reflected in the species of TLR9. Certainly, the ultimate proof will be to demonstrate by biochemical means whether CpG-DNA directly binds to TLR9.

6. DNA-PKcs AS A CRITICAL MOLECULE FOR CpG-DNA-INDUCED CELL ACTIVATION

Also employing cells from gene deficient animals, another molecule, the DNA-dependent protein kinase catalytic subunit “DNA-PKcs,” has been found to be involved in the responsiveness of innate immune cells towards CpG-DNA (30). Originally, DNA-PKcs has been characterized as the catalytic subunit of DNA-PK, an enzyme complex composed of the regulatory factor “KU,” consisting in a heterodimer of Ku70 and Ku80 and the DNA-PKcs protein (for review see 66). DNA-PK has an essential function in the repair of DNA double-strand breaks, which can be provoked by exogenous DNA-damaging agents, but also originate as physiological intermediates in the process of V(D)J-recombination of T and B cells. Therefore, one of the consequences of loss or widely reduced levels (like in SCID-mice) of DNA-PK activity is the failure to produce mature T and B cells. Primarily ends of dsDNA seem to activate DNA-PK, however, at high concentrations and below a length of 20 bases, binding and activation of DNA-PKcs by single-stranded ODNs has also been demonstrated (67). In that study, sequence-specificity has not been investigated.

The key finding of the report of Chu et al. is that macrophages from DNA-PK-deficient mice show a substantial defect in CpG-DNA-induced IKK and NF- κ B activation, accompanied by an almost complete loss of IL-12 and IL-6 production (30). Notably, these cells exhibit no alteration in their responses toward LPS stimulation. Obviously, DNA-PKcs-deficient macrophages have a specific loss—or reduction—in their ability to respond to CpG-DNA. Moreover, DNA containing unmethylated CpG-motifs was found preferentially to activate DNA-PK (human DNA-PKcs complexed with Ku70 and Ku80) in vitro. In vivo, this effect seems to be less obvious than as demonstrated for bacterial double-stranded DNA (30). Using recombinant proteins, DNA-PK has been suggested to phosphorylate directly IKK β , leading to activation of the IKK complex and NF- κ B translocation.

Taken together, these results raise the interesting possibility that CpG-DNA is transported to an intracellular compartment where it is recognized

by and activates DNA-PK (or DNA-PKcs), ultimately resulting in NF- κ B activation.

As yet, one would have to speculate when attempting to put together the various observations about TLR9 and DNA-PKcs to draw a solid and unifying model of CpG-DNA-induced cell activation. At first, it seems to be necessary to address some fundamental questions experimentally. For example, are all signaling pathways initiated by CpG-DNA abolished in DNA-PKcs-deficient cells—as in TLR9-deficient cells—or is it rather a selective loss of NF- κ B activation? Is DNA-PKcs activated by CpG-DNA only in cells that are responsive to CpG-DNA, i.e., specialized immune cells? Is the species specificity, contained in certain sequences of CpG-ODN (mouse vs human) reflected in the corresponding species of DNA-PK? Notably, one intriguing idea has been put forward with regard to the receptors involved in LPS-signaling. It is well established that TLR4 is critically involved in LPS-stimulation of immune cells (68,69). Very recently, two other molecules, Nod1 and Nod2 have also been implicated in LPS-signaling. These cytoplasmic molecules that show homology to so-called disease resistance (R) proteins of plants contain a N-terminal caspase recruitment domain (CARD) as effector domain, a centrally located nucleotide-binding domain and C-terminal leucine-rich repeats (LRR). The LRR have been shown to confer binding to LPS, as it has been suggested for the LRR of TLR4 (70). Nod1 seems to be expressed primarily in epithelial cells (71), whereas Nod2 is restricted to monocytes (72). Transfected in naturally non-responsive cells, Nod1 and Nod2 renders these cells LPS-responsive (70). Therefore, it is possible that different cellular compartments and different cell types bear particular receptors to gain responsiveness to a particular PAMP like LPS. It is conceivable that TLR9 and DNA-PK reflect a similar situation for CpG-DNA-recognition. Certainly, a large number of other possibilities must be considered at this stage, ranging from the idea of a sequentially operating signaling pathway, where one molecule depends on the other, to the possibility that gene deficiency (in knock-out animals) results in complex side effects, impinging on CpG-DNA-responsiveness.

7. CpG-DNA REQUIRES ENDOCYTOSIS AND ENDOSOMAL MATURATION TO ACTIVATE IMMUNE CELLS

Beside its unusual biochemical structure, CpG-DNA bears another peculiarity in that it is not a cell wall component of pathogens like most other PAMPs, but is contained in the interior of microorganisms and hidden from direct recognition by the immune system. It has been interesting to see that agents that block endosomal acidification and hence maturation interfere with the stimulatory capacity of CpG-DNA (23,43,73). In the context of

physiological situations, this could mean that the receptor system for CpG-DNA lies downstream of cell wall destruction and consecutive liberation of the pathogen's DNA, a process that usually takes place in the lysosomal compartment of innate immune cells. Using GFP-tagged MyD88 to see where recruitment and—probably—engagement of TLR9 takes place, a vesicular pattern in CpG-DNA-stimulated cells appears, consistent with the idea that signaling via MyD88 initiates at an endosomal compartment (unpublished observations). Certainly, with respect to the finding that cytoplasmic DNA-PKcs represents a critical molecule for CpG-DNA-dependent cell activation, the endosomal compartment as signaling compartment must be interpreted cautiously as it might be only one, but not the exclusive site where signaling is initiated. If endocytosis of CpG-DNA—physiologically in the context of complete pathogens—is required for signal induction, then the uptake process might be immunologically more significant than for other PAMPs. Moreover it is interesting in an evolutionary sense. Until now, it has been argued that CpG-DNA, like other foreign substances, must be structurally different and distinguishable from the vertebrate's host DNA to meet a dogma in the immune system, i.e., the discrimination of self from nonself. With respect to bacteria and vertebrates this seems to be the case as DNA of these organisms substantially differ in their content of unmethylated CGs and probably in the content of unmethylated stimulatory CpG-motifs. For *Drosophila* and its genome, which harbors immunostimulatory potency, it would mean that DNA is no reasonable candidate to serve as a target of the immune system. However, if stimulatory DNA is recognized in an intracellular compartment (endosomal or cytoplasmic) after liberation from its covering shell, the decision which structures can be used as a PAMP might be made by receptor systems anywhere in the pathway from the cell surface to the intracellular signaling compartment. Determining the evolutionary stages of the CpG-DNA-recognition system(s), will be one of the most interesting topics of the near future.

8. SUMMARY AND CONCLUSION

CpG-DNA activates a plethora of signaling events in cells of the innate immune system like DCs, macrophages, and B cells. Cell activation is preceded by endocytosis of CpG-DNA and endosomal maturation to reach a cellular compartment where signaling is initiated (Fig. 3). Two molecules, DNA-PKcs, a cytoplasmic protein and TLR9, presumably a transmembrane protein, have been proposed to be critically involved in recognition of CpG-DNA. The interconnection of these proteins has not yet been defined. For TLR9, endosomal uptake and probably processing of bacterial DNA could suffice to provide the appropriate ligand to initiate signal transduction via

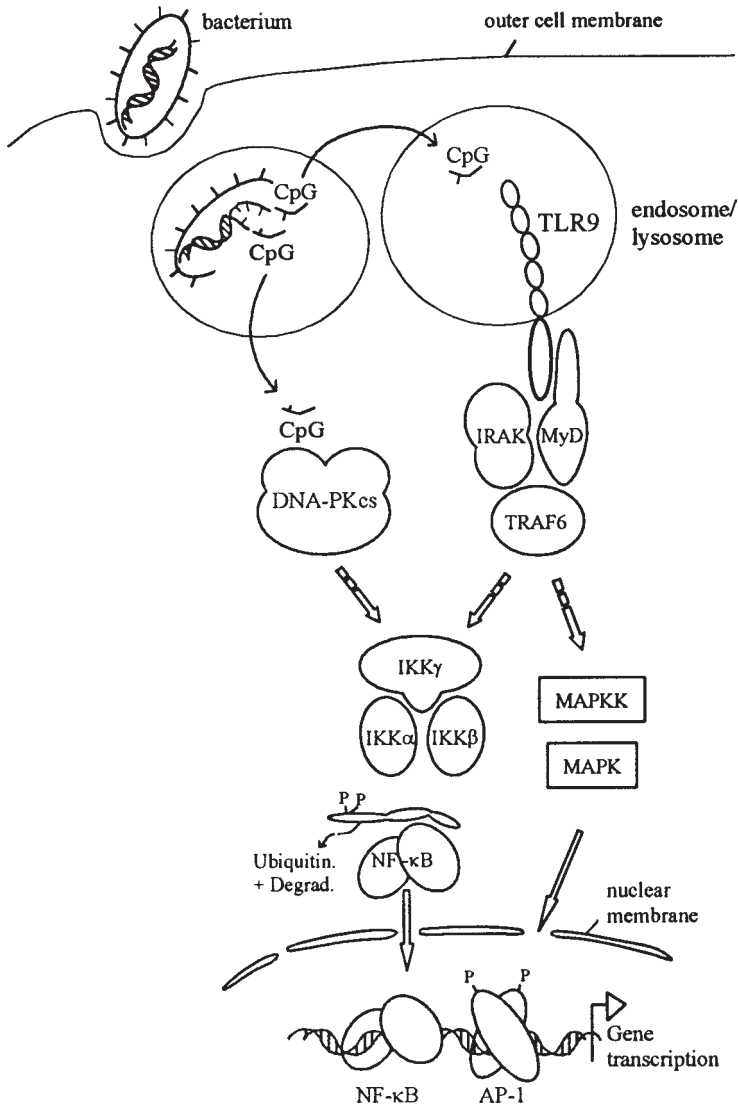


Fig. 3. Model of CpG-DNA-induced signal transduction. For abbreviations see Fig. 1.

engagement of MyD88 and TRAF6, components of the TLR/IL-1R-pathway, followed by activation of the IKK complex and different MAP kinase pathways, ultimately resulting in activation of transcription factors like NF- κ B and AP-1. In contrast to TLR9, direct binding and activation of DNA-PKcs seems to require transfer of CpG-DNA over the membrane barrier, followed

by phosphorylation of IKK β and activation of NF- κ B. Although the data about DNA-PKcs and TLR9 have extended our understanding of the molecular mechanism of CpG-DNA, many new questions have emerged: Does CpG-DNA directly bind to TLR9 and DNA-PKcs? What is the molecular mechanism of the species specificity contained in different stimulatory DNA-sequences? What is the contribution of the two molecules, DNA-PKcs and TLR9, to the different signaling cascades activated and where is the point of convergence? How and where is double-stranded bacterial DNA processed to reach and bind its receptor systems? Nevertheless, the identification of CpG-DNA-specific key molecules justifies the hope that it will be possible to define the role of stimulatory DNA in the physiological context of infection and immunity.

REFERENCES

1. Tokunaga, T., Yamamoto, T., and Yamamoto, S. (1999) How BCG led to the discovery of immunostimulatory DNA. *Jpn. J. Infect. Dis.* **52**, 1–11.
2. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O. and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN- mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.
3. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
4. Wagner, H. (1999) Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol.* **73**, 329–368.
5. Takeuchi, O., Hoshino, K., Kawai, T., et al. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443–451.
6. Means, T. K., Golenbock, D. T., and Fenton, M. J. (2000) The biology of Toll-like receptors. *Cytokine. Growth Factor. Rev.* **11**, 219–232.
7. Wesche, H., Henzel, W.J., Shillinglaw, W., Li, S. & Cao, Z. (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**, 837–847.
8. Ishida, T., Mizushima, S., Azuma, S., et al. (1996) Identification of TRAF6, a novel tumor necrosis factor receptor- associated factor protein that mediates signaling from an amino- terminal domain of the CD40 cytoplasmic region. *J. Biol. Chem.* **271**, 28,745–28,748.
9. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev.* **13**, 1297–1308.
10. Ghosh, S., May, M. J., and Kopp, E. B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16**, 225–260.

11. Karin, M. and Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity (2000). *Annu. Rev. Immunol.* **18**, 621–663.
12. Rothwarf, D. M., Zandi, E., Natoli, G. and Karin, M. (1998) IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature* **395**, 297–300.
13. Yamaoka, S., Courtois, G., Bessia, C., et al. (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **93**, 1231–1240.
14. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F., and Verma, I. M. (1999) Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science*, **284**, 321–325.
15. Li, Z. W., Chu, W., Hu, Y., et al. (1999) The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J. Exp. Med.* **189**, 1839–1845.
16. Hu, Y., Baud, V., Delhase, M., et al. (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* **284**, 316–320.
17. Karin, M. and Delhase, M. (1998) JNK or IKK, AP-1 or NF-kappaB, which are the targets for MEK kinase 1 action? *Proc. Natl. Acad. Sci. USA* **95**, 9067–9069.
18. Yin, L., Wu, L., Wesche, H., et al. (2001) Defective lymphotoxin-beta receptor-induced NF-kappaB transcriptional activity in NIK-deficient mice. *Science* **291**, 2162–2165.
19. Deng, L., Wang, C., Spencer, E., et al. (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103**, 351–361.
20. Inohara, N., Koseki, T., Lin, J., et al. (2000) An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* **275**, 27,823–27,831.
21. Poyet, J. L., Srinivasula, S. M., Lin, J. H., et al. (2000) Activation of the Ikappa B kinases by RIP via IKKgamma /NEMO-mediated oligomerization. *J. Biol. Chem.* **275**, 37,966–37,977.
22. Stacey, K. J., Sweet, M. J. and Hume, D. A. (1996) Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116–2122.
23. Yi, A. K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J., and Krieg, A. M. (1998) CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* **160**, 4755–4761.
24. Sparwasser, T., Miethke, T., Lipford, G., et al. (1997) Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. *Eur. J. Immunol.* **27**, 1671–1679.
25. Yi, A. K., Klinman, D. M., Martin, T. L., Matson, S., and Krieg, A. M. (1996) Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* **157**, 5394–5402.
26. Baeuerle, P. A. and Henkel, T. (1994) Function and activation of NF-kappa B in the immune system. *Annu. Rev. Immunol.* **12**:141-79, 141–179.

27. Muroi, M., Muroi, Y., and Suzuki, T. (1994) The binding of immobilized IgG2a to Fc gamma 2a receptor activates NF- kappa B via reactive oxygen intermediates and tumor necrosis factor- alpha 1. *J. Biol. Chem.* **269**, 30,561–30,568.
28. Muller, J. M., Rupec, R. A. and Baeuerle, P. A. (1997) Study of gene regulation by NF-kappa B and AP-1 in response to reactive oxygen intermediates. *Methods* **11**, 301–312.
29. Hacker, H., Vabulas, R. M., Takeuchi, O., et al. (2000) Immune Cell Activation by Bacterial CpG-DNA through Myeloid Differentiation Marker 88 and Tumor Necrosis Factor Receptor-Associated Factor (TRAF)6. *J. Exp. Med.* **192**, 595–600.
30. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs Is Required for Activation of Innate Immunity by Immunostimulatory DNA. *Cell* **103**, 909–918.
31. Murphy, T. L., Cleveland, M. G., Kulesza, P., Magram, J., and Murphy, K. M. (1995) Regulation of interleukin 12 p40 expression through an NF-kappa B half- site. *Mol. Cell Biol.* **15**, 5258–5267.
32. Plevy, S. E., Gemberling, J. H., Hsu, S., Dorner, A. J. and Smale, S. T. (1997) Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell Biol.* **17**, 4572–4588.
33. Sanjabi, S., Hoffmann, A., Liou, H. C., Baltimore, D., and Smale, S. T. (2000) Selective requirement for c-Rel during IL-12 P40 gene induction in macrophages. *Proc. Natl. Acad. Sci. USA* **97**, 12,705–12,710.
34. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40.
35. Whitmarsh, A. J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R. J. (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. *Science* **281**, 1671–1674.
36. Schaeffer, H. J., Catling, A. D., Eblen, S. T., Collier, L.S., Krauss, A., and Weber, M. J. (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. *Science* **281**, 1668–1671.
37. Roussel, M. F. (1998) Key effectors of signal transduction and G1 progression. *Adv. Cancer Res.* **74**, 1–24.
38. Sluss, H. K., Barrett, T., Derijard, B. and Davis, R. J. (1994) Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell Biol.* **14**, 8376–8384.
39. Westwick, J. K., Weitzel, C., Minden, A., Karin, M., and Brenner, D. A. (1994) Tumor necrosis factor alpha stimulates AP-1 activity through prolonged activation of the c-Jun kinase. *J. Biol. Chem.* **269**, 26,396–26,401.
40. Bird, T. A., Kyriakis, J. M., Tyshler, L., Gayle, M., Milne, A. and Virca, G. D. (1994) Interleukin-1 activates p54 mitogen-activated protein (MAP) kinase/stress-activated protein kinase by a pathway that is independent of p21ras, Raf-1, and MAP kinase kinase. *J. Biol. Chem.* **269**, 31,836–31,844.
41. Saklatvala, J., Davis, W. and Guesdon, F. (1996) Interleukin 1 (IL1) and tumour necrosis factor (TNF) signal transduction. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **351**, 151–157.

42. Hambleton, J., Weinstein, S. L., Lem, L., and DeFranco, A. L. (1996) Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *Proc. Natl. Acad. Sci. USA* **93**, 2774–2778.
43. Hacker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.
44. Karin, M., Liu Zg, and Zandi, E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol.* **9**, 240–246.
45. Smeal, T., Binetruy, B., Mercola, D., et al. (1992) Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. *Mol. Cell Biol.* **12**, 3507–3513.
46. Sanchez, I., Hughes, R. T., Mayer, B. J., Yee, K., Woodgett, J. R., Avruch, J., Kyriakis, J. M., and Zon, L. I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. *Nature* **372**, 794–798.
47. Gupta, S., Campbell, D., Derijard, B. and Davis, R. J. (1995) Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* **267**, 389–393.
48. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell Biol.* **16**, 1247–1255.
49. Yi, A. K., Chang, M., Peckham, D. W., Krieg, A. M., and Ashman, R. F. (1998) CpG oligodeoxynucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J. Immunol.* **160**, 5898–5906.
50. Sanghera, J. S., Weinstein, S. L., Aluwalia, M., Girn, J., and Pelech, S. L. (1996) Activation of multiple proline-directed kinases by bacterial lipopolysaccharide in murine macrophages. *J. Immunol.* **156**, 4457–4465.
51. Hacker, H., Mischak, H., Hacker, G., et al. (1999) Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. *EMBO J.* **18**, 6973–6982.
52. Sester, D. P., Beasley, S. J., Sweet, M. J., et al. (1999) Bacterial/CpG DNA down-modulates colony stimulating factor-1 receptor surface expression on murine bone marrow-derived macrophages with concomitant growth arrest and factor-independent survival. *J. Immunol.* **163**, 6541–6550.
53. Buscher, D., Hipskind, R. A., Krautwald, S., Reimann, T. and Baccarini, M. (1995) Ras-dependent and -independent pathways target the mitogen-activated protein kinase network in macrophages. *Mol. Cell Biol.* **15**, 466–475.
54. Geppert, T. D., Whitehurst, C. E., Thompson, P., and Beutler, B. (1994) Lipopolysaccharide signals activation of tumor necrosis factor biosynthesis through the ras/raf-1/MEK/MAPK pathway. *Mol. Med.* **1**, 93–103.
55. Yi, A. K. and Krieg, A. M. (1998) Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493–4497.
56. Lu, H. T., Yang, D. D., Wysk, M., Gatti, E., Mellman, I., Davis, R. J., and Flavell, R. A. (1999) Defective IL-12 production in mitogen-activated protein (MAP) kinase kinase 3 (Mkk3)-deficient mice. *EMBO J.* **18**, 1845–1857.

57. Winzen, R., Kracht, M., Ritter, B., et al. (1999) The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J.* **18**, 4969–4980.
58. Prichett, W., Hand, A., Sheilds, J., and Dunnington, D. (1995) Mechanism of action of bicyclic imidazoles defines a translational regulatory pathway for tumor necrosis factor alpha. *J. Inflamm.* **45**, 97–105.
59. Feng, G. J., Goodridge, H. S., Harnett, M. M., et al. (1999) Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J. Immunol.* **163**, 6403–6412.
60. Medzhitov, R. and Janeway, C. (2000) The Toll receptor family and microbial recognition. *Trends. Microbiol.* **8**, 452–456.
61. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
62. Schnare, M., Holt, A. C., Takeda, K., Akira, S., and Medzhitov, R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* **10**, 1139–1142.
63. Du, X., Poltorak, A., Wei, Y., and Beutler, B. (2000) Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur. Cytokine. Netw.* **11**, 362–371.
64. Chuang, T. H. and Ulevitch, R. J. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. (2000) *Eur. Cytokine. Netw.* **11**, 372–378.
65. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
66. Smith, G. C. and Jackson, S. P. (1999) The DNA-dependent protein kinase. *Genes Dev.* **13**, 916–934.
67. Hammarsten, O., DeFazio, L. G. and Chu, G. (2000) Activation of DNA-dependent protein kinase by single-stranded DNA ends. *J. Biol. Chem.* **275**, 1541–1550.
68. Poltorak, A., He, X., Smirnova, I., et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085–2088.
69. Hoshino, K., Takeuchi, O., Kawai, T., et al. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* **162**, 3749–3752.
70. Inohara, N., Ogura, Y., Chen, F. F., Muto, A., and Nunez, G. (2001) Human Nod1 Confers Responsiveness to Bacterial Lipopolysaccharides. *J. Biol. Chem.* **276**, 2551–2554.
71. Inohara, N., Koseki, T., del Peso, L., et al. (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J. Biol. Chem.*, **274**, 14,560–14,567.

72. Ogura, Y., Inohara, N., Benito, A., Chen, F. F., Yamaoka, S., and Nunez, G. (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF{ κ }B. *J. Biol. Chem.*, **276**, 2551–2554.
73. Macfarlane, D. E. and Manzel, L. (1998) Antagonism of immunostimulatory CpG-oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. *J. Immunol.* **160**, 1122–1131.

A Novel Toll-Like Receptor that Recognizes Bacterial DNA

Hiroaki Hemmi and Shizuo Akira

1. INTRODUCTION

In mammalian immune system, activation of innate immunity is crucial for protecting the host from invading pathogens. The innate immune system specifically recognizes structural patterns of microbial components, which are conserved among a wide variety of microorganisms. These structural patterns are termed pathogen associated molecular patterns (PAMPs), and include lipopolysaccharide (LPS), peptidoglycan (PGN), bacterial lipoproteins (BLP), mannans, and bacterial DNA (1). All of these are essential for the microbe's survival and are not expressed on the host. The host immune system has developed the receptors that can specifically recognize PAMPs, so-called pattern recognition receptors (PRRs). As one of PRRs, Toll-like receptor (TLR) family has been well studied (2,3). TLR family has been phylogenetically conserved from insects to mammals and characterized as a type I transmembrane protein with leucine-rich repeats in the extracellular domain and a cytoplasmic Toll/interleukin (IL)-1 receptor homology (TIR) domain. So far, ten members of human TLR are identified (2). Biological functions of some TLR family members are revealed; TLR4 is responsible for the recognition of LPS and TLR2 is involved in that of PGN and BLP (3). The activation of TLRs induces recruitment of the adaptor protein MyD88 and sequential activation of signaling molecules such as IRAK (IL-1 receptor associated kinase) and TRAF6 (TNF receptor associated factor). Finally, transcription factors AP-1 and NF- κ B are activated and translocated to the nucleus, where they induce gene expression of cytokines such as IL-1 β , IL-6, IL-12 p40, and costimulatory molecules such as CD80 and CD86.

It has been reported that MyD88 is essential for activation of immune cells in response to CpG DNA (4,5). MyD88-deficient cells show neither

proliferative response of splenocytes nor production of inflammatory cytokines from macrophages in response to CpG DNA. It also has been shown that TLR2- or TLR4-deficient cells normally responded to CpG DNA (5). These results suggested that other TLR family member(s) might be necessary for recognition of CpG DNA.

We identified a novel TLR family member, TLR9, by using a BLAST search. To investigate physiological function(s) of TLR9, we generated TLR9-deficient (TLR9^{-/-}) mice and analyzed the immune responses to various bacterial products, including immunostimulatory DNA containing CpG motifs (6). We examined proliferative response of splenocytes and inflammatory cytokine production from macrophages. In addition to these studies in vitro, we tested whether CpG DNA-mediated lethal shock and Th1-like response were also defective in TLR9^{-/-} mice in vivo. We also investigated the response to guanosine-rich oligodeoxynucleotides (ODN) that does not contain CpG motifs but can induce proliferation of splenocytes or bone marrow cells (BMCs).

3. RESULTS AND DISCUSSION

3.1. Identification of Novel Toll-Like Receptor

To identify novel TLRs, we performed a BLAST search using TLR4 as a query. We identified an expressed sequence tagged clone (GenBank accession number: AA272731) that showed high similarity with previously identified TLRs. We isolated a full-length cDNA by using this fragment as a probe. We also isolated the human counterpart. Sequence analysis revealed the presence of regions conserved in TLR family, such as LRR and TIR domain. So, we named this gene TLR9. Northern blot analysis of various tissues showed that TLR9 transcripts were abundantly expressed in bone marrow and the spleen.

To assess the biological function(s) of TLR9, we generated TLR9^{-/-} mice by homologous recombination in embryonic stem cells (6). TLR9^{-/-} mice were born at expected mendelian-ratio, and did not show any abnormal morphology. Flow cytometric analysis showed that TLR9^{-/-} mice have normal composition of lymphocytes.

3.2. Cellular Response to CpG DNA is Defective in TLR9^{-/-} Cells

Bacterial DNA has immuno-stimulatory effects on mammalian immune cells, which is responsible for the presence of unmethylated CpG dinucleotides in particular base contexts (7–9). It is known that a lot of ODN containing different CpG motifs can activate mammalian immune cells, particularly human and mouse immune cells are optimally stimulated by

slightly different CpG motifs, 5'-GTCGTT-3' and 5'-GACGTT-3' for human and mouse, respectively (9). ODN 1668 has one CpG motif, 5'-GACGTT-3', which possess strong immunogenic activity for mouse immune cells (10), and ODN 2006 contains three CpG motifs, 5'-GTCGTT-3', optimal for activating human cells (11). AAC-22 has one CpG motifs, 5'-AACGTT-3', which can stimulate mouse immune cells. Control ODN does not contain any CpG motifs (12).

Splenocytes from wild-type or TLR9^{-/-} mice were stimulated with these three CpG ODNs. All of these CpG ODNs induced proliferation of wild-type splenocytes in a dose dependent manner (Fig. 1A). In contrast, TLR9^{-/-} splenocytes did not proliferate in response to these CpG ODNs as well as control ODN, although they showed a similar proliferative response to that of wild-type cells in response to LPS (Fig. 1A). We also measured production of inflammatory cytokines from peritoneal macrophages stimulated with a combination of interferon (IFN)- γ and ODNs by enzyme-linked immunosorbent assay (ELISA). Wild-type peritoneal macrophages produced tumor necrosis factor (TNF)- α , IL-12 p40, and IL-6 in response to CpG ODNs containing ODN 1668, ODN 2006, and AAC-22, but not to control ODN (Fig. 1B and data not shown). On the other hand, production of these cytokines from TLR9^{-/-} macrophages was not detected when stimulated with either these CpG ODNs or control ODN, although they did produce TNF- α in response to LPS or PGN (Fig. 1B). Moreover, TLR9^{-/-} peritoneal macrophages did not respond to bacterial DNA derived from *Escherichia coli* even in the presence of IFN- γ (Fig. 1B). These results indicate that TLR9 were essential for response to CpG DNA.

Next, we investigated whether or not the activation of the intracellular signaling cascade in response to CpG ODN were induced in TLR9^{-/-} cells. Stimulation of TLRs leads to the sequential recruitment of MyD88 and the serine/threonine kinase IL-1 receptor associated kinase (IRAK), subsequently activates mitogen-activated protein kinases and the transcription factor NF- κ B (13). It has also been reported that stimulation of CpG DNA causes activation of MAP kinase c-Jun N-terminal kinase (JNK) and transcription factor NF- κ B (14,15). Therefore, we analyzed the activation of signaling cascades in response to CpG DNA. As shown in Fig. 2, stimulation of ODN 1668 increased the DNA-binding activity of NF- κ B in wild-type macrophages, whereas this activation in TLR9^{-/-} macrophages was abolished. In vitro kinase assay showed that activation of JNK and IRAK did not occur in TLR9^{-/-} macrophages in response to ODN 1668. LPS stimulation led to activation of these intracellular molecules to the same extent as that of wild-type cells (6). Thus, CpG DNA-mediated signal transduction is dependent on TLR9.

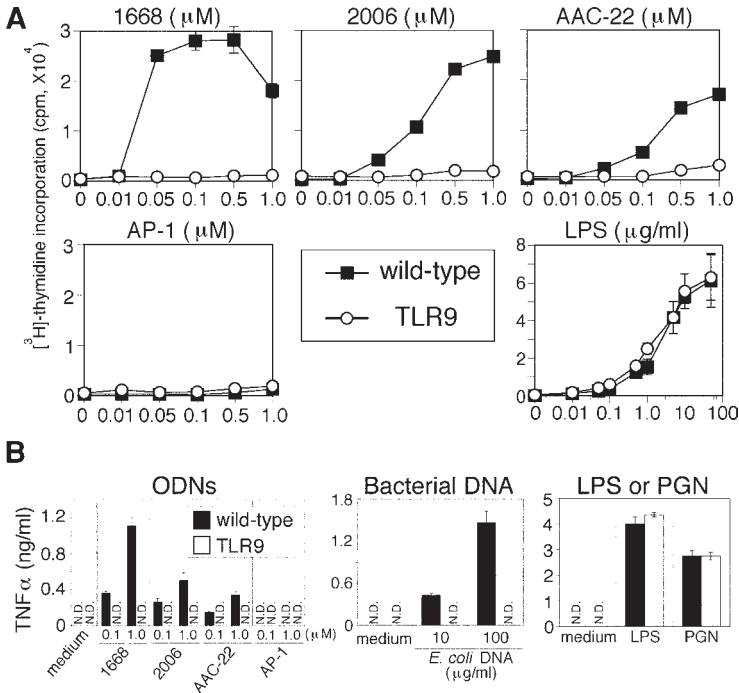


Fig. 1. TLR9^{-/-} cells failed to respond to various CpG DNA. **(A)** Splenocytes from wild-type and TLR9^{-/-} mice were stimulated with indicated concentrations of ODN 1668 (phosphorothioate backbone, 5'-TCCATGACGTTCTCTGATGCT-3'), ODN 2006 (phosphorothioate backbone, 5'-TCGTCGTTTTGTCGTTTTGTC GTT-3'), ODN AAC-22 (phosphorothioate backbone, 5'-ACCGATAACGTTGCC GGTGACG-3'), control ODN (phosphorothioate backbone, 5'-GCTTGATGA CTCAGCCGGAA-3') or LPS for 48 h plus pulsed [³H]thymidine for the last 8 h. [³H]thymidine incorporation was measured by β -scintillation counter. Data indicate mean $\pm$ s.d. **(B)** Thioglycollate-elicited peritoneal macrophages from wild-type or TLR9^{-/-} mice were stimulated with indicated concentrations of ODN 1668, ODN 2006, ODN AAC-22, control ODN, LPS, PGN, or genomic DNA from *E. coli* in the presence of IFN- γ (30 U/mL) for 24 h, and concentrations of TNF- α in the culture supernatants were measured by ELISA. Data indicated mean $\pm$ s.d.

3.3. In Vivo Response to CpG DNA in TLR9^{-/-} Mice

Next, we addressed in vivo response to CpG DNA in TLR9^{-/-} mice. CpG DNA can induce lethal shock in D-galactosamine (D-GalN) -sensitized mice (15). All TLR9^{-/-} mice survived over 120 h and did not show any increase of serum concentration of TNF- α , IL-6, and IL-12, although wild-type mice died within 12 h after administration of D-GalN plus ODN 1668 with marked elevation of serum concentrations of these cytokine levels (6). It has been

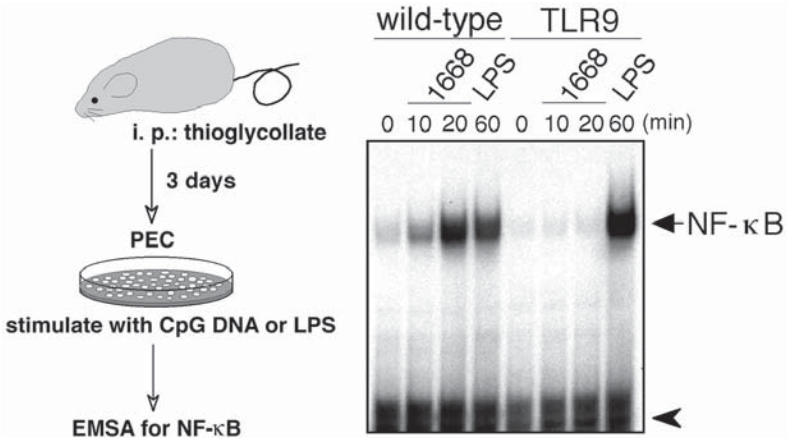


Fig. 2. CpG DNA-induced NF- κ B activation did not occur in TLR9^{-/-} macrophages. Peritoneal macrophages were stimulated with 1.0 μ M ODN 1668 or 1.0 μ g/mL LPS for the indicated durations (left). NF- κ B activity was determined by EMSA (right). Arrow indicates NF- κ B complex; (arrow head) indicates free probes.

reported that CpG DNA act as adjuvant for Th1 based response (16,17). ODN 1668 and ovalbumin (OVA) were injected into the footpads, and popliteal lymph node (LN) cells were collected seven days later and stimulated with OVA. In ODN 1668-treated wild-type mice, popliteal LNs contained the increased number of cells ($23.8 \pm 14.5 \times 10^6$ cells/two popliteal LNs) compared with that of PBS-treated mice ($1.0 \pm 0.2 \times 10^6$ cells/two popliteal LNs), whereas TLR9^{-/-} mice showed no lymphadenopathy (PBS-treated; $1.0 \pm 0.4 \times 10^6$ cells/two popliteal LNs, ODN 1668 plus OVA-treated; $2.3 \pm 0.9 \times 10^6$ cells/two popliteal LNs). Popliteal LN cells from ODN 1668-treated TLR9^{-/-} mice did not produce IFN- γ in response to OVA, although production of IFN- γ from that of wild-type mice was observed⁶. Thus, Th1-like response induced by CpG DNA was also defective in TLR9^{-/-} mice.

3.4. TLR9^{-/-} Cells Normally Responded to Guanosine-Rich ODN

Previous studies have been reported that a 30-mer deoxyguanosine ODN (dG30) containing phosphodiester backbones are mitogenic for splenocytes (18) and ODN GR1, that is a guanosine-rich ODN containing nuclease resistant phosphorothioate backbones, lead to proliferation of macrophage-like cells from bone marrow cells (BMCs) without inducing cytokines or hematopoietic growth factors (19). Next, we investigated whether the proliferative response to these guanosine-rich ODN lacking the CpG motif (G-rich ODN) is also defective, or not in TLR9^{-/-} mice. Splenocytes were stimulated with dG30 for 48 h. Of interest, splenocytes from TLR9^{-/-} mice

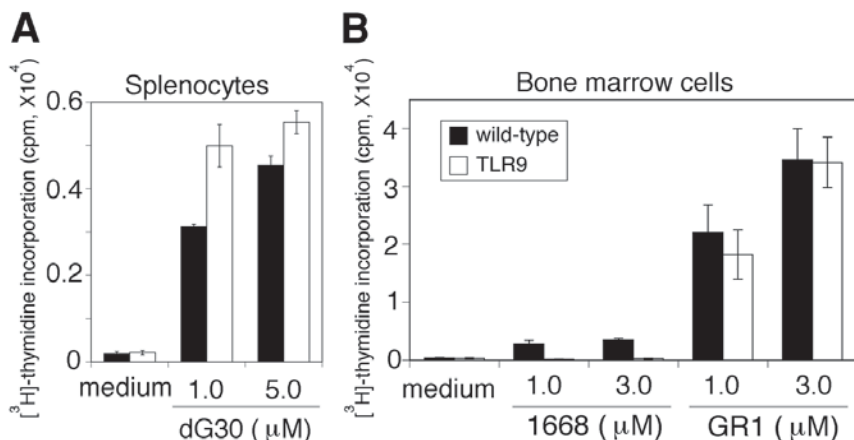


Fig. 3. TLR9^{-/-} cells normally responded to G-rich ODN. **(A)** Splenocytes from wild-type or TLR9^{-/-} mice were stimulated with the indicated concentrations of dG30 for 48 h plus pulsed with [³H]thymidine for the last 8 h. [³H]thymidine incorporation was measured. Data indicated mean ± s.d. **(B)** BMCs from wild-type or TLR9^{-/-} mice were cultured with the indicated concentrations of ODN 1668 or GR1 for six days plus pulsed with [³H]thymidine for the last 8 h [³H]thymidine incorporation was measured. Data indicated mean ± s.d.

showed the same proliferative response as that of wild-type mice (Fig 3A). BMCs were stimulated with ODN 1668 or GR1 for six days. As previously reported, ODN 1668 were less effective to induce proliferation of BMCs than that of GR1 (Fig. 3B). GR1 induced a strong proliferative response of TLR9^{-/-} BMCs, as well as that of wild-type BMCs. These results indicate that TLR9 are not essential for induction of mitogenic response to G-rich ODN, such as GR1 and dG30, and also suggest that the molecular mechanism(s) of cellular activation via G-rich ODN differ from that of ODN containing the CpG motif.

3.5. Perspectives

Recently, it has been reported that DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are necessary for CpG-DNA-mediated activation of immune cells (20). The relationship between TLR9 and DNA-PKcs in the immune response to CpG DNA remains unclear. DNA-PKcs may locate the downstream of signaling cascades initiated from TLR9 activation, or these two molecules may be activated independently in response to CpG DNA and both activation may be necessary for immune response (Fig. 4). Further investigations are required to clarify this point.

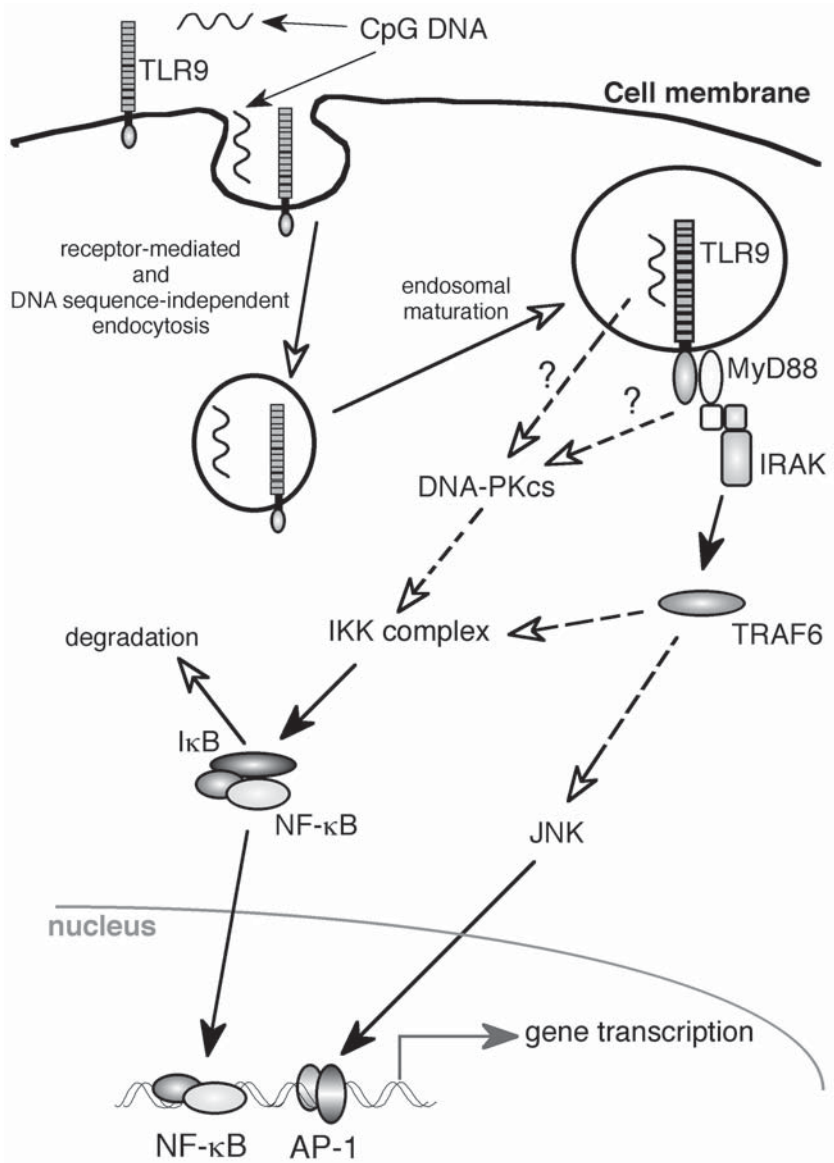


Fig. 4. Model of signaling pathways induced by CpG DNA. *See text for details.*

Activation of immune cells via CpG DNA is shown to require cellular uptake by DNA sequence-independent endocytosis, and endosomal acidification (9,14). Although TLR9 has a transmembrane domain and signal pep-

tides, TLR9 are predicted to be expressed on the cell surface, but not in the cytoplasm, as is the case of other TLR family members. Activation of IRAK, as well as JNK activation, after CpG DNA stimulation was slightly delayed as compared with LPS-induced activation (6). TLR9 may be internalized with CpG DNA or bacteria into endosomes via nonspecific endocytosis and activated after endosomal maturation (Fig. 4). Although further investigations are required, the identification of signaling receptor for CpG DNA will add a new insight to our understandings on the mechanisms of CpG DNA recognition, as well as to therapeutic applications of CpG DNA for cancer, infectious diseases, and allergy.

ACKNOWLEDGMENTS

We thank N. Tsuji for excellent secretarial assistance. This work was supported by grants from Special Coordination Funds of the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

1. Medzhitov, R. and Janeway, C. A., Jr. (1997) Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295–298.
2. Aderem, A. and Ulevitch, R. J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782–787.
3. Kaisho, T. and Akira, S. (2000) Critical roles of Toll-like receptors in host defense. *Crit. Rev. Immunol.* **20**, 393–405.
4. Häcker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., and Wagner, H. (2000) Immune cell activation by bacterial CpG-DNA through myeloid differential marker 88 and tumor necrosis factor receptor-associated factor (TRAF) 6. *J. Exp. Med.* **192**, 595–600.
5. Schnare, M., Holt, A. C., Takeda, K., Akira, S., and Medzhitov, R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* **10**, 1139–1142.
6. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
7. Van Uden, J. and Raz, E. (2000) Introduction to immunostimulatory DNA sequences. *Springer Semin. Immunopathol.* **22**, 1–9.
8. Yamamoto, S., Yamamoto, T., and Tokunaga, T. (2000) The discovery of immunostimulatory DNA sequence. *Springer Semin. Immunopathol.* **22**, 11–19.
9. Kreig, A. M. and Wagner, H. (2000) Causing a commotion in the blood: immunotherapy progresses from bacteria to bacterial DNA. *Immunol. Today* **21**, 521–526.
10. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.

11. Hartmann, G., Weeratna, R. D., Ballas, Z. K., et al. (2000) Delineation of a CpG Phosphorothioate Oligodeoxynucleotide for Activating Primate Immune Responses In Vitro and In Vivo. *J. Immunol.* **164**, 1617–1624.
12. Yamamoto, T., Yamamoto, S., Kataoka, T., and Tokunaga, T. (1994) Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. *Microbiol. Immunol.* **38**, 831–836.
13. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity* **11**, 115–122.
14. Häcker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.
15. Sparwasser, T., Miethke, T., Lipford, G., et al. (1997) Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor- α -mediated shock. *Eur. J. Immunol.* **27**, 1671–1679.
16. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
17. Lipford, G. B., Sparwasser, T., Zimmermann, S., Heeg, K., and Wagner, H. (2000) CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J. Immunol.* **165**, 1228–1235.
18. Pisetsky, D. S., Reich, C. F., 3rd. (1998) The influence of base sequence on the immunological properties of defined oligonucleotides. *Immunopharmacology* **40**, 199–208.
19. Lang, R., Hultner, L., Lipford, G. B., Wagner, H., and Heeg, K. (1999) Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. *Eur. J. Immunol.* **29**, 3496–3506.
20. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.

Activation of Innate Immunity by Microbial Nucleic Acids

Wen-Ming Chu, Xing Gong, and Tony Yoon

1. INTRODUCTION

An effective innate response to microbial product requires the identification of pathogen-associated molecular patterns (PAMPs, e.g., dsRNA, CpG-DNA or LPS), by the host via pattern recognition receptors (PRR; 1,2). Two different recognition systems were established to explain the activation of innate immunity by microbial product. The first PRR is intracellular and utilizes the PKR to recognize dsRNA, an intermediate product during viral replication (3). The second PRR is a cell surface receptor e.g., Toll-like receptors (TLRs) (2,4). TLRs have been reported to interact and to initiate innate response to a variety of microbial products TLR4-LPS, TLR2-peptidoglycan, TLR5-flagelline, and TLR9-ISS-ODN (2,4).

In this chapter, we will briefly discuss immune activation by microbial nucleic acids, and specifically, the role of PKR in activation of NF- κ B by viral dsRNA and the role of DNA-dependent protein kinase (DNA-PKcs) in activation of NF- κ B by bacterial ISS-DNA.

2. PKR AND IKK ARE REQUIRED FOR THE INNATE IMMUNE RESPONSE TO dsRNA

Type 1 interferon (IFNs, i.e., IFN α and IFN β) is a family of proteins with distinct biological properties, the most prominent of which is their ability to impair viral replication. The antiviral effects of type 1 IFNs are in part mediated by the action of PKR. Once activated by dsRNA, PKR autophosphorylates itself and, in turn, phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2 α), leading to a decrease in protein synthesis in the host cells (5).

As type 1 IFNs play a very important role in protection of the host from viral infection, the molecular mechanism of induction of type 1 IFNs by

dsRNA has been intensively investigated. dsRNA treatment was reported to activate NF- κ B and cause secretion of type 1 IFNs in mouse lung cancer fibroblasts (6,7). Examination of IFN β promoter region reported that the NF- κ B and other transcription factors such as c-jun, ATF2 and IFN regulatory factors (IRFs) are required for expression of IFN β (8).

Subsequent studies suggested that PKR is required for activation of NF- κ B by dsRNA because depletion of PKR mRNA impaired NF- κ B activation (9). It was also reported that PKR activates NF- κ B by phosphorylating NF- κ B's inhibitor I κ B α (10). Further studies using PKR-deficient mice confirmed that PKR is required for activation of NF- κ B by dsRNA and induction of type 1 IFNs by dsRNA is diminished in PKR-deficient mouse embryo fibroblasts (MEFs) (11).

The IKK is a physiological kinase complex that phosphorylates I κ Bs, and is composed of two catalytic subunits, IKK α and IKK β , and one regulatory subunit, IKK γ /NEMO (12–16). IKK β and IKK γ /NEMO are required for activation of IKK and NF- κ B by many stimuli. These include the proinflammatory cytokines TNF α , and IL-1 and bacterial lipopolysaccharide (LPS) (16–21). In contrast, IKK α mediates NF- κ B activation to an unidentified developmental signal that controls keratinocyte differentiation (22–25).

Since dsRNA activates NF- κ B, it was hypothesized that the activation of NF- κ B is mediated by IKK (17). Indeed, incubation of MEFs derived from wild-type (WT) mice with dsRNA resulted in a significant increase in NF- κ B and IKK activation, whereas the use of MEFs derived from IKK β -deficient mice severely impaired activation of IKK by dsRNA. In contrast, MEFs derived from IKK α -deficient mice did not affect the activation of IKK by dsRNA (Chu et al., unpublished observation).

To investigate if activation of IKK contributes to induction of type 1 IFNs by dsRNA, we performed a Northern blot assay using WT, IKK α - or IKK β -deficient MEFs. Low levels of IFN α and IFN β were observed in IKK β -deficient MEFs as compared to WT control. By contrast, loss of IKK α subunit had almost no effect on induction of IFN α or IFN β by dsRNA.

Although PKR is required for activation of NF- κ B and induction of type 1 IFNs by dsRNA, we reasoned that PKR is required for activation of IKK by dsRNA (17). Incubation of dsRNA with WT MEFs resulted in activation of IKK and NF- κ B whereas the loss of PKR severely impaired activation of IKK and NF- κ B by dsRNA. Cotransfection of PKR and IKK β into PKR-deficient MEFs restored the activation of IKK by dsRNA.

To further understand how PKR activates IKK, we performed a transient co-transfection assay and an *in vitro* kinase assay. We found that either WT PKR or a dominant negative PKR (296 K to R) activates IKK, suggesting

that the interaction between IKK and PKR is required for activation of IKK. The interaction between PKR and IKK was subsequently confirmed (26–27).

3. IKK IS REQUIRED FOR INNATE IMMUNE RESPONSES TO ISS-DNAs

Bacterial DNA contains multiple repeats of unmethylated CpG motifs (28–30). ISS-DNA and its synthetic ODN analogs (ISS-ODNs), stimulate innate immunity to produce cytokines such as TNF α , IL-6, IL-10, IL-12, IL-18, and type 1 IFN (31). Subsequent studies suggested that ISS-DNA activates the transcription factors NF- κ B and activation protein 1 (AP-1) as a result of activation of c-jun kinase (JNK) and p38 by ISS-DNA (32–34). It has been reported that the ISS-DNA has to be internalized to mediate activation of innate immunity and that ISS-ODN covalently linked to a solid support loses its stimulatory effects on mouse B cells (36).

As discussed previously, activation of NF- κ B by dsRNA is mediated by IKK. We further assumed that activation of NF- κ B by ISS-DNAs is also mediated by IKK (37). To test this possibility, we used different ISS-DNAs such as phosphothioate ISS-ODN, phosphodiester ISS-ODN (po-ISS-ODN), and bacterial DNA. We found that ISS-DNAs significantly induced IKK activation in bone marrow-derived macrophages (BMDM) from WT whereas control DNAs only induced minimal activation of IKK. Moreover, we found that IKK β subunit is critical for activation of IKK and NF- κ B by ISS-DNAs. Furthermore, using BMDM from Tnfr $^{-/-}$ or Tnfr $^{-/-}$ IKK $\beta^{-/-}$ mice, we found that IKK activation significantly contributes to induction of cytokines by ISS-ODN.

4. DNA-PKcs IS REQUIRED FOR INDUCTION OF CYTOKINES BY ISS-DNAs

DNA-PK is a member of phosphatidylinositol 3 (PI3) kinase-like family that also includes ATM, FRAP, and FRP1 (38,39). DNA-PK can be detected within both cytoplasm and nucleus (39–43) (Chu et al., unpublished observation). In the nucleus, DNA-PK plays a pivotal role in repair of DNA double-stranded breaks (DSB) created by environmental results such as ionizing irradiation (38,40,44) or by intrinsic cellular processes such as programmed DNA rearrangements (V.D.J recombination) during lymphocyte differentiation (44). In contrast, the cytoplasmic function of DNA-PK is unknown.

The full function of DNA-PK requires the catalytic subunit of DNA-PK (DNA-PKcs) and the regulatory subunits of Ku70 and Ku80. In response to DNA damaging reagents or γ -irradiation, Ku70 and Ku80 bind to DNA DSBs and then associate with DNA-PKcs, leading to activation of DNA-

PKcs (40,44). Recent evidence suggested that DNA-PKcs directly binds to double- or single-stranded DNA with double-stranded DNA ends (47,48). Like SCID mice DNA-PKcs-, Ku70- or Ku80-deficient mice lack matured B and T cells (49–53).

To test the involvement of DNA-PK in induction of the innate cytokines, IL-6 and IL-12 by ISS-DNAs, we used BMDM from WT or DNA-PKcs-deficient mice (37). Incubation of WT BMDM with ISS-DNAs resulted in inducing high levels of IL-6 and IL-12. However, loss of DNA-PKcs severely impaired induction of IL-6 and IL-12 by ISS-DNAs. In contrast, both WT and DNA-PKcs-deficient BMDM were equally responsive to LPS in producing IL-6 and IL-12. Northern blot assay indicated lower levels of IL-6, and IL-12 transcripts in DNA-PKcs-deficient BMDM as compared to WT control. However, almost the same levels of IL-6 and IL-12 were observed in both wt and DNA-PKcs-deficient BMDM in responding to LPS, suggesting that the lack of response to ISS-DNAs in DNA-PKcs-deficient BMDM occurs at the transcriptional level.

To further explore the requirement of DNA-PKcs for induction of IL-6 and IL-12 by ISS-DNAs *in vivo*, we injected ISS-DNAs and control DNA (calf thymus DNA) to wt or DNA-PKcs-deficient mice. The levels of IL-6 and IL-12 mRNA levels in liver or spleen were determined by RT-PCR. Lower levels of IL-6 and IL-12 were observed in DNA-PKcs-deficient mice as compared to wt control (Fig. 1). As we expected, calf thymus DNA only induced minimal amounts of IL-6 and IL-12 mRNA levels in WT mice (Fig. 1).

DNA-PK is a member of PI3K family and its enzymatic activity is blocked by PI3K inhibitors such as wortmannin (Wm, 39). To further identify the role of DNA-PKcs in the induction of IL-6 and IL-12 by ISS-ODN, we examined the effects of Wm on these responses. We found that Wm at high concentration significantly inhibits the induction of IL-6 and IL-12 by ISS-ODN.

Biological and genetic studies revealed a loss of DNA-PKcs function in severe combined immunodeficiency (SCID) cells (39). This results from a mutation in the DNA-PKcs gene that converts the Tyr-4046 codon into a stop codon, creating a truncated protein missing the last 83 amino acid of the kinase domain (39). Although this mutated protein is highly unstable, it is still present at detectable levels (39,42), raising the possibility that SCID cells retain residual DNA-PKcs function (42,55). Interestingly, the levels of cytokines induced by ISS-ODN are intact in SCID BMDM as compared to their WT controls (55; Chu et al., unpublished observation). To explore this finding, we examined the level of DNA-PK or activation of DNA-PK by ISS-ODN in SCID BMDM, and found a detectable level of DNA-PK and its activity (Chu et al., unpublished observation). Rag1- or Rag2-deficient mice

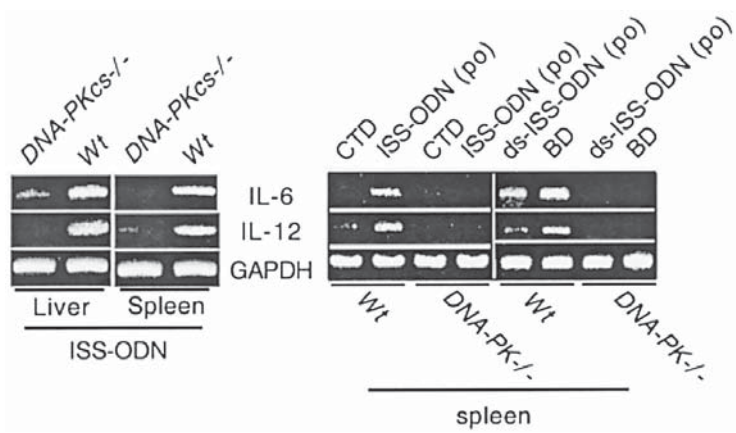


Fig. 1. DNA-PK is required for cytokine induction by ISS-DNA in vivo. Wild-type (wt) or DNA-PKcs-deficient mice were injected with ISS-ODN (20 μ g/mouse), po-ISS-ODN (20 μ g/mouse) or bacteria DNA (BD, 50 μ g/mouse) or calf thymus DNA (CTD, 50 μ g/mouse). After 3 h, mice were sacrificed and total RNA was isolated from liver or spleen and then subjected to RT-PCR analysis.

have similar phenotypes to SCID mice. The response of BMDM from Rag1- or Rag2-deficient mice to ISS stimulation was assessed. BMDM from these mice mount a similar cytokine response to ISS-DNA as was observed for BMDM from WT mice (Chu et al., unpublished observation).

5. ISS-DNA ACTIVATES DNA-PK

To test the hypothesis that ISS-ODN activates DNA-PK, we performed an in vitro kinase assay using affinity-purified DNA-PK and its substrate, p53, in the presence or absence of ISS-ODN. ISS-ODN strongly activated DNA-PK in vitro. The activation of DNA-PK by ISS-DNA was evaluated in BMDM. WT or DNA-PKcs-deficient BMDM were incubated with ISS-DNAs, the DNA-PK complex was immunoprecipitated, and DNA-PK kinase activity was determined. We found that ISS-DNAs significantly activate DNA-PK in WT, but not in DNA-PKcs-deficient BMDM (Fig. 2a).

We next evaluated the involvement of DNA-PKcs in activation of IKK by ISS-DNAs. Thus, IKK activation by ISS-DNAs was studied in both WT and DNA-PKcs-deficient BMDM. The loss of DNA-PKcs subunit impaired both IKK and NF- κ B by ISS-DNAs (Fig. 2b). To further clarify how DNA-PK is involved in activation of IKK by ISS-DNA, we performed an in vitro kinase assay using affinity-purified DNA-PK and recombinant IKK α and IKK β purified from insect cell lysates. Incubation of DNA-PK with IKK in

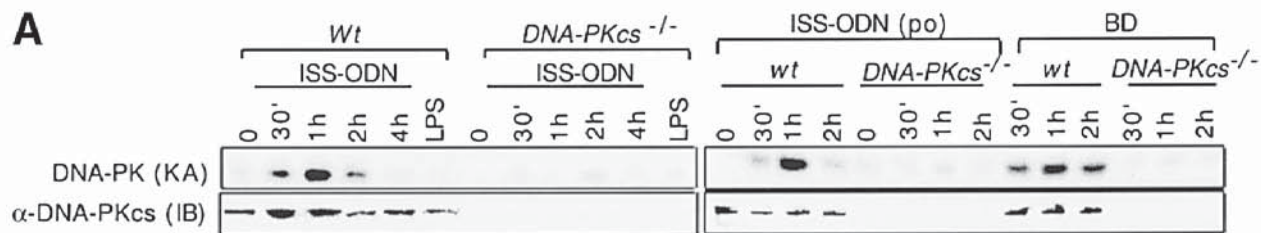


Fig. 2a. ISS-DNA activates DNA-PK BMDM from wt or DNA-PKcs-deficient mice were treated with ISS-DNAs [ISS-ODN (5 ug/ml), ISS-ODN-po (5μg/mL) or bacterial DAN (BD, 5 μg/mL)] or LPS (10 μg/mL, 30 min) for the indicated time, and then lysed. One hundred ug of cell lysates were incubated with anti-DNA-PKcs mAb (Pharmegien) and protein A beads for overnight at 4°C. The DNA-PKcs complex was washed and DNA-PKcs kinase activity was determined by a kinase assay using GST-p53 (1–70) as a substrate. The equal amount of protein was normalized by an immunoblotting assay using anti-DNA-PKcs mAb (BD-PharMegien).

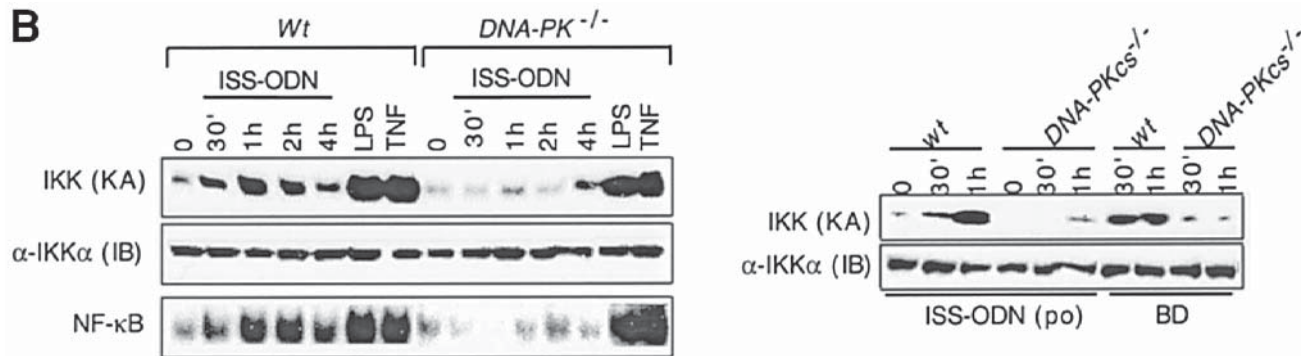


Fig. 2b. DNA-PKcs is required for activation of IKK and NF-κB by ISS-DNAs. BMDM from wt or DNA-PKcs – deficient mice were treated with ISS-DNAs or LPS as described above or TNF (10 ng/mL, for 10 min). IKK kinase complex was immunoprecipitated using anti-IKKα antibodies (Santa Cruz Biotech.) and its kinase activity was determined by as kinase assay using GST-IκBα (1–54) as a substrate. The presence of IKK in lysates was normalized by an immunoblotting assay using anti-IKKα antibodies.

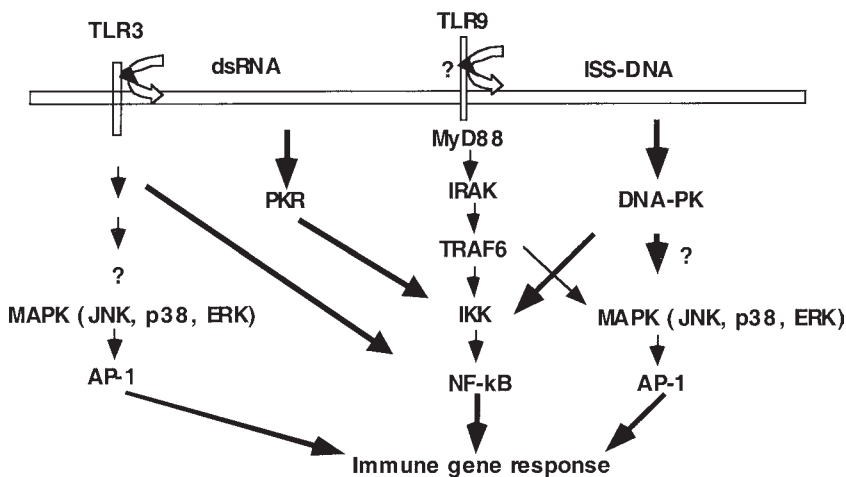


Fig. 3. Molecular Mechanisms of Activation of Innate Immunity by dsRNA and ISS-DNA. Activation of IKK and NF- κ B by dsRNA is mediated by PKR (17). It is most likely that another molecule (e.g., TLR3) is involved in the dsRNA signaling pathway, and serve as a cell membrane receptor for dsRNA (56). Akira and Wagner's groups reported that Toll-like receptor (TLR) 9 and adaptor protein MyD88 are required for activation of innate immunity by ISS-ODN (57). The possible interaction of TLR pathway with DNA-PK is given (37,57).

the presence of ISS-ODN significantly increased IKK β but not IKK α kinase activity. To further confirm this result, we preincubated IKK with DNA-PK in the presence or absence of ISS-ODN, immunoprecipitated IKK from the reaction and then determined IKK kinase activity. In the presence of ISS-ODN, DNA-PK significantly activated IKK.

In addition, we used recombinant dominant negative IKK β (K to A) as a substrate for DNA-PK to show that DNA-PK phosphorylates IKK β . As we expected, DNA-PK phosphorylated IKK β (37).

6. FUTURE PROSPECTS

As presented in Subheading 5. and Fig. 3, DNA-PKs and PKR are involved in activation of IKK and NF- κ B by ISS-DNA and dsRNA, respectively. The activation of JNK by dsRNA is not mediated by PKR (17), suggesting an additional signaling pathway. Indeed, recent studies suggested that TLR3 is required for activation of NF- κ B by dsRNA (56, Chu et al. unpublished data), suggesting a possibility of TLR3 involvement in activation of mitogen-activated protein kinases (MAPKs) such as ERK, JNK, and p38. However, further evidence of showing if TLR3 is a receptor for dsRNA should be collected.

On the other hand, it has been reported that ISS-DNA also activates MAPKs (33). Future studies will identify if DNA-PKcs is involved in activation of the MAPK pathway. In addition, the role of the two regulatory subunits, Ku70 and Ku80 of DNA-PK in mediating activation of innate immunity by ISS-DNA should be assessed. These studies will further clarify the relationship between DNA repair machinery and innate immunity.

The innate immune system has evolved to recognize PAMPs. PKR and DNA-PKcs were shown to play a role in the recognition of dsRNA and ISS-DNA, respectively. As described previously in this volume, TLR9 was recently shown to mediate the ISS-DNA signaling pathway (4,57). However, the relationship between DNA-PK and TLR9 is not immediately apparent. Further studies will need to elucidate the potential interaction between TLR9 and DNA-PK, or to further define their separate signaling pathways.

ACKNOWLEDGMENTS

We thank Dr E. Raz for his support and helpful comments. This work was supported by NIH grant AI40682, a grant from Dynavax Technologies Corporation, and support from the Office of the President, University of California.

REFERENCES

1. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. J., and Ezekowiz, R. A. B. (1999) Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318.
2. Aderem, A., Ulevitch, R. J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782–787.
3. Patton, J. T., Spencer, E. (2001) Genome replication and packaging of segmented double-stranded RNA viruses. *Virology* **277**, 217–225.
4. Akira, S., Takeda, K., Kaisho, T. (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* **2**, 675–680.
5. Chong, K. L., Feng L, Schappert, K., et al. (1992) Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator GCN2. *EMBO J.* **11**, 1553–1562.
6. Lenardo, M. J, Fan, C.M, Maniatis, T., and Baltimore, D. (1989) The involvement of NF-kappa B in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. *Cell.* **57**, 287–294.
7. Visvanathan, K. V., and Goodbourn S. (1989) Double-stranded RNA activates binding of NF-kappa B to an inducible element in the human beta-interferon promoter. *EMBO J.* **8**, 1129–1138.
8. Du, W., Maniatis, T. (1992) An ATF/CREB binding site is required for virus induction of the human interferon beta gene. *Proc. Natl. Acad. Sci. USA* **89**, 2150–2154.
9. Maran, A., Maitra, R. K., Kumar, A., et al. (1994). Blockage of NF-kappa B signaling by selective ablation of an mRNA target by 2-5A antisense chimeras. *Science.* **265**, 789–792.

10. Kumar, A., Haque, J., Lacoste, J., Hiscott, J., Williams, B. R. (1994) Double-stranded RNA-dependent protein kinase activates transcription factor NF-kappa B by phosphorylating I kappa B. *Proc. Natl. Acad. Sci. USA* **91**, 6288–6292.
- and Bonnet, M. C., Weil, R., Dam, E., Hovanessian, A. G., Meurs, E. F. (2000) PKR stimulates NF-kappaB irrespective of its kinase function by interacting with the IkappaB kinase complex. *Mol. Cell Biol.* **20**, 4532–4542.
11. Yang, Y.-L., Reis, L. F., Pavlovic, J., et al. (1995) Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* **14**, 6095–6106.
12. Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91**, 243–252.
13. Zandi, E., Chen, Y., and Karin, M. (1998) Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. *Science* **281**, 1360–1363.
14. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature* **395**, 297–300.
15. Yamaoka, S., Courtois, G., Bessia, C., et al. (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **93**, 1231–1240.
16. Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999) Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science* **284**, 309–313.
17. Chu, W.-M., Ostertag, D., Li, Z.-W., et al. (1999). JNK2 and IKKbeta are required for activating the innate response to viral infection. *Immunity*. **11**, 721–731.
18. Li, Z.-W., Chu, W.-M., Hu, Y., et al. (1999). The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. *J. Exp. Med.* **189**, 1839–1845.
19. Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F., and Verma, I. M. (1999) Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science*. **284**, 321–325.
20. Tanaka, M., Fuentes, M. E., Yamaguchi, K., et al. (1999) Embryonic lethality, liver degeneration, and impaired NF-kB activation in IKK- β -deficient mice. *Immunity* **10**, 421–429.
21. Karin, M. and Delhase, M. (2000) The I-kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. *Semin Immunol.* **12**, 85–98.
22. Hu, Y., Baud, V., Delhase, M., et al. (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* **284**, 316–312.
23. Hu, Y., Baud, V., Oga, T., Kim, K.I., Yoshida, K., and Karin, M. (2001). IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature* **410(6829)**, 710–714.

24. Li, Q., Lu, Q., Hwang, J. Y., et al. (1999) IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev.* **13**, 1322–1328.
25. Takeda, K., Takeuchi, O., Tsujimura, T., et al. (1999) Limb and skin abnormalities in mice lacking IKK α . *Science* **284**, 313–316.
26. Bonnet, M. C., Weil, R., Dam, E., Hovanessian, A. G., and Meurs, E. F. (2000) PKR stimulates NF- κ B irrespective of its kinase function by interacting with the IkappaB kinase complex. *Mol. Cell Biol.* **20**, 4532–4542.
27. Gil, J., Alcamí, J., Esteban, M. (2000) Activation of NF- κ B by the dsRNA-dependent protein kinase, PKR involves the I κ B kinase complex. *Oncogene* **19**, 1369–1388.
28. Tokunaga, T., Yamamoto, H., Shimada, S., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–662.
29. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992). Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated [correction of INF] natural killer activity. *J. Immunol.* **148**, 4072–4076.
30. Krieg, A. M., Yi, A. K., Matson, S., et al. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation, *Nature* **374**, 546–549.
31. Van Uden, J., and Raz, E. (1999). Immunostimulatory DNA and applications to allergic disease. *J. Allergy Clin. Immunol.* **104**, 902–910.
32. Stacey, K. J., Sweet, M.J., Hume, D.A. (1996) Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116–2122.
33. Hacker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.
34. Yi, A. K., Krieg, A. M. (1998) CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I κ B α and I κ B β and sustained activation of nuclear factor- κ B/c-Rel. *J. Immunol.* **160**, 1240–1245.
35. Yi, A. K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J., and Krieg, A. M. (1998) CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* **160**, 4755–4761.
36. Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S., and Lipsky, P. E. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
37. Chu, W.-M., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
38. Hartley, K. O., Gell, D., Smith, G. C., et al. (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* **82**, 849–856.
39. Smith, G. C., Jackson, S. P. (1999). The DNA-dependent protein kinase. *Genes Dev.* **13**, 916–934.

40. Gottlieb, T. M., and Jackson, S. P. (1993) The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell* **72**, 131–142.
41. Danska, J. S., Holland, D. P., Mariathasan, S., Williams, K. M., Guidos, C. J. (1996) Biochemical and genetic defects in the DNA-dependent protein kinase in murine scid lymphocytes. *Mol. Cell Biol.* **10**, 5507–5517.
42. Koike, M., Awaji, T., Kataoka, M., et al. (1999). Differential subcellular localization of DNA-dependent protein kinase components Ku and DNA-PKcs during mitosis. *J. Cell Sci.* **112**, 4031–4039.
43. Nilsson, A., Sirzen, F., Lewensohn, R., Wang, N., and Skog, S. (1999). Cell cycle-dependent regulation of the DNA-dependent protein kinase. *Cell Prolif.* **32**, 239–248.
44. Kirchgessner, C. U., Patil, C. K., Evans, J. W., et al. 1995 DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science*. **267**, 1178–1183.
45. Mimori, T., and Hardin, J. A. (1986) Mechanism of interaction between Ku protein and DNA. *J. Biol. Chem.* **261**, 10,375–10,379.
46. Leuther, K. K., Hammarsten, O., Kornberg, R. D., and Chu, G. (1999) Structure of DNA-dependent protein kinase: implications for its regulation by DNA. *EMBO J.* **18**, 1114.
47. Hammarsten, O., DeFazio, L. G., and Chu, G. (2000) Activation of DNA-dependent protein kinase by single-stranded DNA ends. *J. Biol. Chem.* **275**, 1541–1545.
48. Nussenzweig, A., Chen, C., Da Costa Soares, V., et al. (1996). Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature* **382**, 551–555.
49. Zhu, C., Bogue, M. A., Lim, D. S., Hasty, P., and Roth, D. B. (1996) Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell*. **86**, 379–389.
50. Gu, Y., Seidl, K. J., Rathbun, G. A., et al. (1997) Growth retardation and leaky SCID phenotype of Ku70-deficient mice. *Immunity*. **7**, 653–665.
51. Manis, J. P., Gu, Y., Lansford, R., et al. (1998). Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J. Exp. Med.* **187**, 2081.
52. Kurimasa, A., Ouyang, H., Dong, L. J., et al. (1999). Catalytic subunit of DNA-dependent protein kinase: impact on lymphocyte development and tumorigenesis. *Proc. Natl. Acad. Sci. USA* **96**, 1403–1408.
53. Li, G. C., Ouyang, H., Li, X., et al. (1998). Ku70: a candidate tumor suppressor gene for murine T cell lymphoma. *Mol. Cell* **2**, 1–8.
54. Carroll, A. M., Bosma, M. J. (1991). T-lymphocyte development in scid mice is arrested shortly after the initiation of T-cell receptor delta gene recombination. *Genes Dev.* **8**, 1357–1366.
55. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344.

56. Alexopoulou, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732–738.
57. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.

Phosphorothioate Backbone Modification to Changes the Pattern of Responses CpG Motifs

Katryn J. Stacey, David P. Sester, Shalin Naik,
Tara L. Roberts, Matthew J. Sweet, David A. Hume

1. INTRODUCTION

Bacterial DNA is immunostimulatory due to the presence of specific unmethylated CpG-containing sequences (1). The activity of bacterial DNA can be mimicked by oligonucleotides (ODN) (2), and this has been critical in establishing the sequence requirements for activation. Both native phosphodiester oligonucleotides and phosphorothioate-modified oligonucleotides (PO-ODN and PS-ODN) of various sequences can activate macrophages, dendritic cells, and B lymphocytes. Although PO-ODN are the most relevant to the role of bacterial or viral DNA in the host response to infection, stabilized synthetic oligonucleotides have great potential in immunotherapy. Normal phosphodiester oligonucleotides are short-lived in vivo (3) and incapable of giving effective therapeutic immunostimulation. The most frequent means of stabilizing oligonucleotides is by phosphorothioate modification of the backbone, whereby one of the nonbridging oxygens of the phosphate group is converted to sulfur. Phosphorothioates are poor substrates for most cellular nucleases. CpG PS-ODN display many of the activities of bacterial DNA, but owing to evidence of phosphorothioate-specific activity, caution has been urged in their use as a model of bacterial DNA in infection (4,5). Although PS-ODN are now widely used in studies on the immunostimulatory effects of CpG-containing DNA, it is appropriate to ask whether they mimic all the actions of native phosphodiester DNA. In this article, we review literature on activities of PS-ODN and discuss our own comparisons of the action of PS- and PO-ODN on macrophage activation.

2. RESULTS AND DISCUSSION

2.1. Relative Potency of CpG PS- and PO-ODN

Assuming that extracellular or intracellular degradation of PO-ODN limits their biological activity, stabilization of the backbone in PS-ODN would be expected to increase potency in assays *in vitro*. Few studies have examined sequence-identical PS- and PO-ODN, but in most cases PS-ODN are found to be active at much lower concentration than PO-ODN. In assays of B-cell proliferation, PS-ODN have been found more potent (4,6–8), but interpretation of this is complicated by CpG-independent responses of B cells to PS-ODN (9,10). In addition, the use of thymidine incorporation as a measure of proliferation in these assays may give an underestimate of PO-ODN activity due to dilution of the radioactive thymidine pool by degraded ODN (11). Analysis of CD69 and IL-12 expression by spleen cells avoids some of these problems and still showed higher potency of PS-ODN (4). Similarly in an assay of natural killer (NK) cell activation (12), induction of IL-6 from spleen cells (13) and survival of dendritic cell precursors from peripheral blood (14) PS-ODN were more effective, although in the latter case the ODN length and sequence were not identical. By contrast, PS- and PO-ODN were found to have similar dose responses in the induction of MHC ClassII, and IL-12p40 in fetal skin-derived dendritic cells (15).

In some cases, the PS modification has been found to eliminate ODN activity. In work on type 2 dendritic cell precursors from human blood (CD4⁺IL-3R α ^{high} CD3⁺CD11c⁻ cells), a PS-ODN that was active in inducing costimulatory molecules failed to induce IFN- α , whereas a PO-ODN did so (16). Strangely, the authors concluded this was probably a sequence-related phenomenon, since the sequences used were not identical, and yet they stated that when the active PO-ODN was made as a PS-version it was still inactive. However, a number of instances do suggest that the effects of PS-modification can be sequence-specific (4,8,17–21). When looking at induction of costimulatory molecule B7-2 in human B cells, Hartmann et al. (18) found that a CpG ODN containing a run of deoxycytidine (dC) residues was active only in the native PO form, whereas with another sequence assessed in the same assay, PS-ODN was more active than the corresponding PO-ODN. This suggests that the sequence requirements for activation by PS-ODN are more stringent (11), and could reflect constraints on oligonucleotide conformation introduced by the phosphorothioate modification. The lack of activity of the aforementioned PS-ODN could be related specifically to the oligo dC region, as Mannon et al. (19) found oligo dC or dG regions flanking an active motif in a PS-ODN were much less favourable

Table 1
Sequences of ODN

AO-1	GCTCATGACGTTTCCTGATGCTG	CpG PO-ODN
AOS-1	GCTCATGACGTTTCCTGATGCTG	CpG PS-ODN
NAO-1	GCTCATGAGCTTCCTGATGCTG	non-CpG PO-ODN
NAOS-1	GCTCATGAGCTTCCTGATGCTG	non-CpG PS-ODN

than oligo dA or dT. Experiments on activation of NK cells determined that runs of dG residues greatly reduced activity when placed within a CpG PS-ODN (20), and yet the same sequence as a PO-ODN or with a chimeric backbone where the CpG motif was phosphodiester was quite stimulatory (20,22). In another study of IL-12 and TNF- α induction in splenic dendritic cells, the PS modification made a CpG ODN much more active, and yet had little effect on the same ODN with an added 3' terminal dG₆ sequence (21). Taken together, these studies suggest that the responses to ODN are complex because the cells may respond to several different aspects of the ligand—the CpG motif, the phosphorothioate backbone or oligo dG. Runs of dG residues are also known to have CpG-independent effects (5,19,21,23,24).

We have carried out extensive comparisons of relative efficacy of different forms of ODN on mouse macrophages. As observed in several other functional assays above, PS-ODN were more active than PO-ODN at low concentrations in a number of assays. For example, we have shown elsewhere that the mouse macrophage cell line RAW264 cell line responds well to CpG DNA (25), and will produce the toxic mediator nitric oxide (NO) if primed with interferon- γ (26). In an assay of NO production PS-ODN AOS-1 was active at approximately a 30-fold lower dose than the corresponding PO-ODN (Table 1 and Fig. 1) (27). ODN in which the active CpG was reversed to GpC had negligible activity (Figure 1). As another assay of CpG activity, we have used a stably-transfected RAW264 cell line in which the endothelial cell-leukocyte adhesion molecule (ELAM) promoter (28), drives expression of a green fluorescent protein (GFP) reporter gene. This promoter requires activation of the transcription factor complex, NF- κ B, and the cell line provides a convenient assay of CpG DNA responses. PS-ODN were substantially more active at low concentration than PO-ODN of identical sequence in activating the ELAM promoter (Fig. 2). We also found previously that PS-ODN were more potent in activation of the IL-12 promoter driving luciferase in RAW264 cells (27). We have commonly used murine bone marrow-derived macrophages (BMM) in studies of CpG DNA responses (25,27,29). These cells require the growth factor CSF-1 for the maintenance of viability. In previous work, we have found

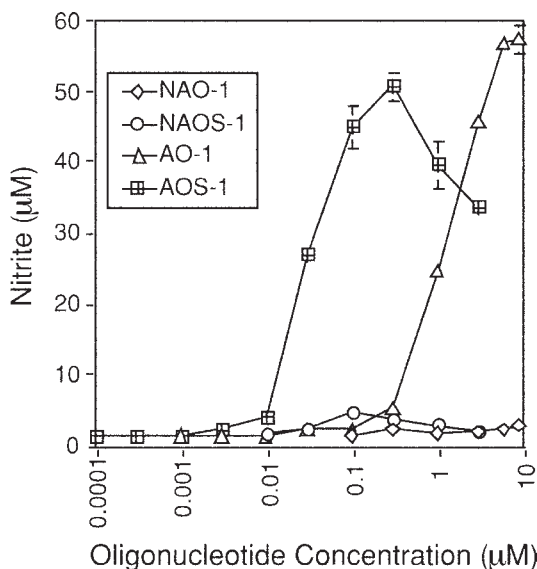


Fig. 1. PS-ODN are more potent than PO-ODN in induction of macrophage NO production (27). RAW264 cells were treated for two hours with 20U/mL IFN- γ , followed by incubation with various concentrations of PO-ODN (AO-1, NAO-1) or PS-ODN (AOS-1, NAOS-1) for 24 h as described (27). Nitrite was assayed in the supernatant as an indication of NO production as described (26). Data are the average and SD of triplicates. Figure reproduced with copyright permission from Sester et al. (27), (copyright 2000. The American Association of Immunologists).

that CpG-containing ODN can prevent cell death in BMM deprived of the growth factor CSF-1 (29). PS-ODN were again more effective than PO-ODN in preventing apoptosis under these circumstances (27). In all these assays, the PS modification did not increase the maximal level of stimulation, but decreased the concentration required for maximal activity. Powerful activation by PS-ODN at low concentrations may be due to a combination of stability and enhanced uptake (*see Subheading 3.3.*), although PS-specific signaling enhancing the CpG response is also a possibility.

Given the potent activity of PS-ODN in the assays of macrophage activation discussed so far, it came as a surprise that they were minimally active in several shorter term assays. We have shown previously that treatment of CSF-1-starved BMM with LPS or CpG DNA caused synchronous internalization of the CSF-1 receptor (29). This may be associated with the growth inhibition caused by these compounds. The internalization of CSF-1R after

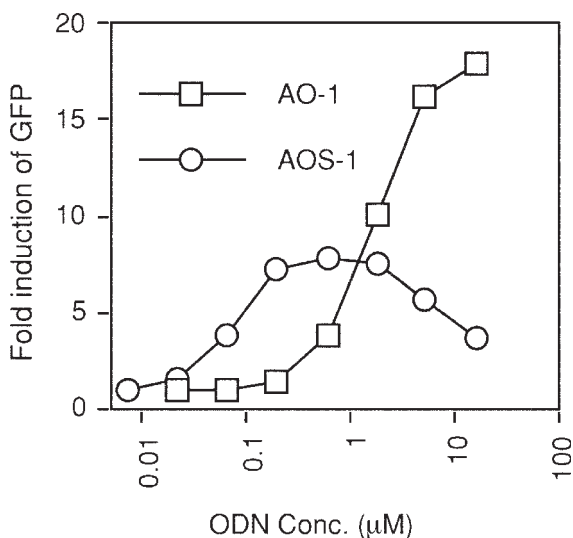


Fig. 2. PS-ODN are more potent than PO-ODN in induction of the ELAM promoter in RAW264 cells. The human ELAM promoter driving destabilized GFP reporter (Clontech) was obtained from K.Smith (Institute for Sytems Biology, Seattle, WA). This was stably integrated into RAW264 cells and an isolated clone with highly inducible GFP expression was selected for use. Cells were plated in a 24-well plate at 250,000 cells/well in 0.5 mL RPMI medium overnight. PS- and PO-ODN were added at various concentrations for four hours before harvesting in cold PBS with 1 mM EDTA and 0.1% sodium azide. GFP expression was analyzed by FACS. The data presented are the fold induction of the mean level of fluorescence of the ODN-treated cell population compared with untreated cells.

one hour of treatment can be used as an assay for CpG-dependent activity of *E.coli* DNA or PO-ODN. In this assay, the PS-ODN AOS-1 gave a barely detectable response (Fig. 3). There was some evidence of the sequence-dependence noted previously above in that a PS-ODN of different sequence had slightly better activity, but it remained weak (27). PS-ODN had a similar low activity in stimulation of a further reporter gene, the HIV-1 LTR driving luciferase stably integrated into RAW264 cells (27).

Both assays showing a relatively poor response to PS-ODN, were assays with an early peak response to CpG DNA (1–2 h). Although we have previously shown reasonable induction of the inflammatory cytokine TNF- α in response to PS-ODN by two hours (27), more recent work suggests that the PS-ODN effect in this assay is very time-dependent. A two hour incubation

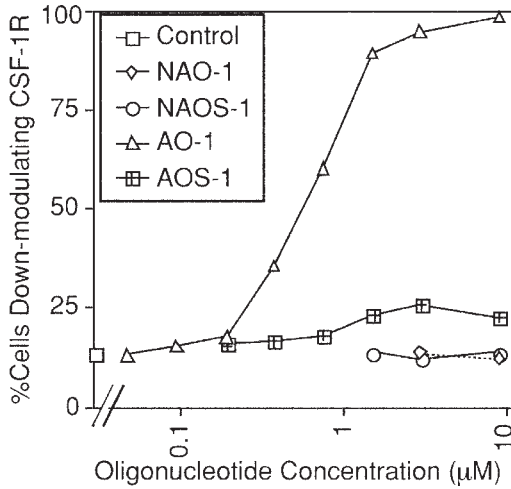


Fig. 3. PS-ODN are not effective at removing cell surface CSF-1R (27). BMM were starved of CSF-1 overnight, then incubated with the indicated concentrations of ODN for one hour and immunostained for surface expression of CSF-1R as described (29). Figure reproduced with copyright permission from Sester et al. (27). Copyright 2000. The American Association of Immunologists

is just on the boundary of detection of PS-ODN responses, and longer times show a much greater relative potency of PS-ODN. These observations suggest a delay in the response to PS-ODN, and we therefore examined early signaling responses to CpG ODN.

3.2. Effects of PS-ODN on Early Signaling Events

The MAPKinases p38 and JNK are activated by CpG DNA and LPS, and activation of ERK-1/2 seems to occur in a cell-specific manner (29–31). The timecourse of response to DNA lags a little behind LPS, probably reflecting the requirement for uptake of DNA signaling (D. Sester, unpublished). Phosphorylation of p38 correlates with its kinase activity and can be readily monitored by Western blot. In RAW264 cells we found that p38 activation was maximal by 20 min after addition of PO-ODN AO-1 (Fig. 4). By contrast, with PS-ODN phosphorylation of p38 was delayed and reached a lower peak at one h. We also observed a delay in activation of ERK-1/2 by PS-ODN, and found similar results in BMM for both MAPKinases ([27] and unpublished results). The delay in signaling provides a straightforward explanation for the

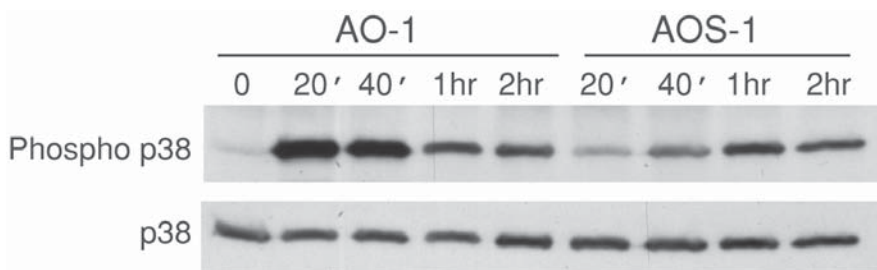


Fig. 4. Activation of p38 by PS-ODN is delayed. RAW264 cells were treated with 3 μM AO-1 and 0.3 μM AOS-1 (optimal stimulatory concentrations) and extracts prepared at various times for Western Blotting as described (29). The blot was probed with anti-phospho p38, stripped and reprobed with anti-p38 antibody as a loading control.

inability to detect PS-ODN responses in short term assays. Some responses may require a large burst of signaling activity and do not occur in its absence.

Others have reported evidence of early MAPKinase activation by PS-ODN. Häcker et al. (30) found stimulation of ERK-2 kinase activity in RAW264 cells, measured by immune complex kinase assay, to peak at 30 min after exposure to PS-ODN. The same workers also showed rapid activation of p38 and JNK 1/2 by PS-ODN in bone marrow-derived dendritic cells. Because these workers did not study corresponding PO-ODN, there is no way of knowing whether unmodified DNA would have been more rapid. In support of the delayed signaling model, LPS induced NF- κ B in a human myeloma cell line after 30 min, but there was no response to PS-ODN until two hours (32). There was a corresponding delay in the decay of the inhibitor of NF- κ B, I κ BB, in the same cells in response to PS-ODN. Work on the IL-12 promoter suggested that PS-ODN induced binding of a nonstimulatory NF- κ B complex to IL-12 promoter within 30 min, but a stimulatory p50/c-Rel complex was not bound until after four hours (33). Despite this, similar responses to PS- and PO-ODN in activation of I κ B kinase, which phosphorylates I κ B and targets it for degradation, have been reported in BMM (34). However, in this work the PS- and PO-ODN treatments did not seem to be done in parallel, and the authors used a PO-ODN concentration, which we find suboptimal in most assays.

Under our experimental conditions, the effect of delayed signaling by PS-ODN can be seen in a time course of activation of the ELAM promoter (Fig. 5). Both *E. coli* DNA and AO-1 gave readily detectable expression of the GFP reporter at two hours, whereas the same level of expression was not

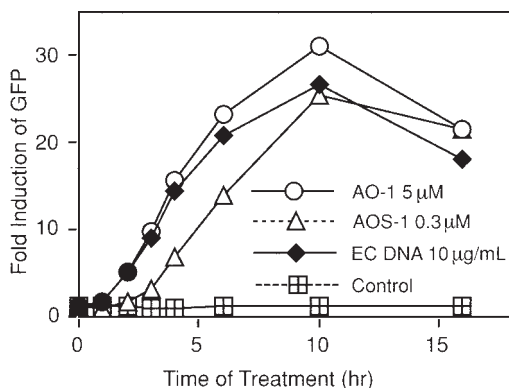


Fig. 5. PS-ODN give delayed activation of the ELAM promoter. RAW264 cells with stably integrated ELAM promoter-GFP reporter were plated and assayed as per Fig. 2. Cells were treated for various times with 0.3 μ M AOS-1, 5 μ M AO-1 or 10 μ g/mL *E.coli* DNA, which elicit maximal responses (Fig. 2). Results are expressed relative to an unstimulated 0 time control.

achieved until about 3.5 h with an optimal concentration of the PS-ODN AOS-1. The response to PS-ODN continued to lag behind the phosphodiester DNAs. Since the bulk of the evidence suggests that CpG responses require DNA uptake (11), we next investigated whether this difference in timecourse of activation reflected different rates of ODN uptake.

3.3. PS-ODN are Rapidly Taken Up by Macrophages

The relative rates of uptake of PO- and PS-ODN were measured by flow cytometric analysis of cells incubated with fluorescently labeled ODN. PS-ODN was clearly taken up much more efficiently than PO-ODN (Fig. 6) (27). This difference was apparent even after five min incubation, prior to any potential problems of PO-ODN degradation and elimination of the fluorophore from the cell. Other studies have shown PS-ODN uptake is either faster (35,36) or slower (37) than PO-ODN, depending on cell type. Our results show that the greater potency of PS-ODN seen in long-term assays in macrophages is likely to be owing not only to higher stability, but also to enhanced cellular accumulation of PS-ODN. However, the deficient early signaling we have observed with PS-ODN cannot be accounted for by lack of uptake. The possibility remains that PS-ODN may be slow in getting to the site of interaction with the putative CpG receptor. Studies by Tonkinson and Stein (38) on HL60 cells have suggested that the intracellular fate of PO- and PS-ODN is different, with PS-ODN reaching a more

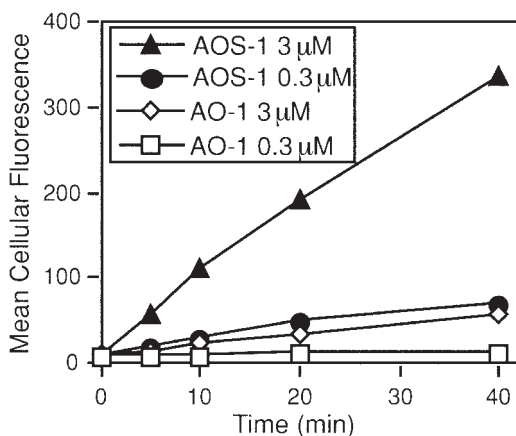


Fig. 6. PS-ODN are taken up more rapidly than PO-ODN (27). RAW264 cells were plated at 250,000/well in one mL of medium overnight. Cy-3 labeled PS- and PO-ODN were added at 0.3 μ M and 3 μ M for various times. Cells were harvested and surface-bound ODN removed with dextran sulfate as described (27). Levels of cell-associated fluorescent ODN were determined by flow-cytometric analysis. Figure reproduced with copyright permission from Sester et al. (27), (copyright 2000. The American Association of Immunologists).

acidified vesicular compartment. Such studies are of course complicated by concerns over the degradation of PO-ODN and separation of label from ODN, especially as the cells were incubated with ODN for six hours.

At least at low concentrations, DNA uptake is believed to be primarily receptor mediated (39,40). The difference we have observed in uptake kinetics suggests that PS-ODN have either a higher affinity for a cell surface receptor than PO-ODN, or bind additional receptors. Our preliminary analysis suggests that PS-ODN binding to the RAW264 cell surface at 4°C is indeed much higher than PO-ODN. The nature of uptake receptors for DNA is still unknown. Beltinger et al. (39) detected at least five cell surface PS-ODN binding proteins. The integrin MAC-1 (CD11b/CD18) has been implicated as an ODN receptor in neutrophils (41), but must there must be other proteins on other cell types because ODN uptake is apparently normal in MAC-1 deficient mice (11).

3.4. PS-ODN Have Inhibitory Activity

Examination of dose response curves of PS-ODN suggests that high concentrations have an inhibitory activity. In a range of assays we find a peak of activity around 0.1–1 μ M with a declining activity at higher concentrations

(Fig. 1 and 2) (27). A clear optimal CpG PS-ODN concentration has been observed by other workers (8,10,19). Inhibitory activity of ODN can be checked by using increasing concentrations of non-CpG ODN, along with an activating stimulus. Pisetsky and Reich (23) found that PS-ODN homopolymers inhibited spleen cell IL-12 production in response to *E.coli* DNA. Of the analogous PO-ODN, only oligo dG was inhibitory. We investigated whether PS-ODN are inhibitory at high concentration using nonstimulatory ODN with inversion of the active CpG. Inactive PS-ODN above 0.3 μ M, but not PO-ODN, was able to inhibit responses to active PS- and PO-ODN in assays of NO production and HIV LTR activity (27). Here we show that inactive PS-ODN NAOS-1 inhibited the number of TNF- α producing RAW264 cells in response to both PS- and PO-ODN (Fig. 7A). Interestingly, at 3 μ M CpG PO-ODN, but not PS-ODN was able to partially overcome this inhibition. This may be because higher concentrations of PS-ODN, whether CpG or non-CpG, have inhibitory action, as seen in Fig. 1 and 2. The inhibitory effect is specific for CpG responses, as LPS response was not affected (Fig. 7B) (27). This rules out a nonspecific toxic effect of the PS-ODN in this experiment. Other workers (23) found that homo-polymer PS-ODN had little effect on the LPS response in BMMs, but confusingly, could inhibit induction of IL-12 in response to LPS in J774 cells.

In previous work, Häcker et al. (42) showed specific inhibition of the response to CpG ODN by non-CpG PS-ODN, and inferred using fluorescence microscopy that the inhibitory ODN prevented uptake of labeled ODN. We have examined uptake of labeled PS- and PO-ODN following pretreatment with non-CpG ODN, and find that although nonstimulatory PS-ODN up to 9 μ M cause some inhibition of uptake, this does not correlate with the degree of inhibition of activity (29). Hence, there must be other inhibitory activities. Because LPS signaling is relatively unaffected in our hands, the PS-ODN is unlikely to have a general effect on inflammatory signaling. Rather it may be interfering with DNA-specific activation, for example by interfering with intracellular localization of the active ODN, or blocking an early step in CpG DNA signaling.

3.5. Backbone-Specific Effects may be Due to Enhanced Protein Binding

As well as the inhibitory activities already detailed, sequence-independent activation by PS-ODN has been observed. In vivo administration of PS-ODN caused some degree of splenomegaly and B cell proliferation in the absence of CpG motifs (9). PS-ODN-mediated enhancement of TNF- α production in response to LPS (43) and sequence-independent activation of human B cells (10) are thought to be mediated by interaction with cell sur-

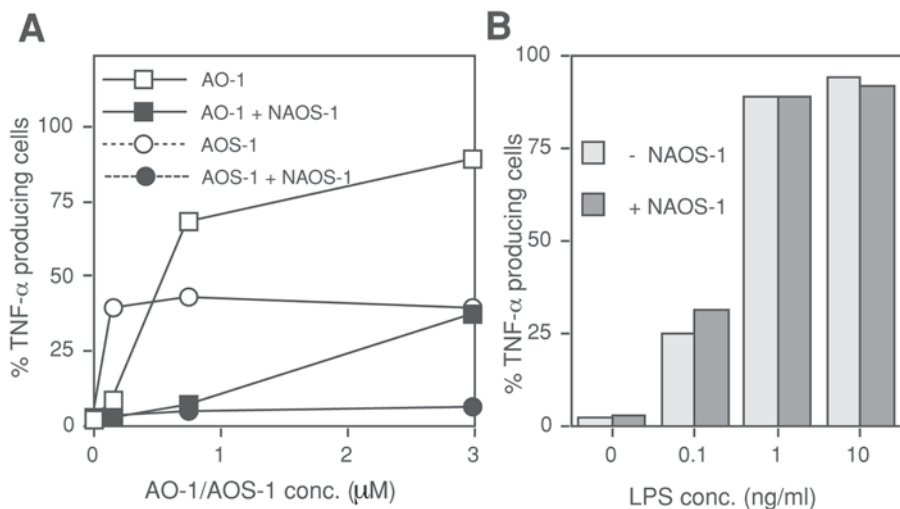


Fig. 7. Inhibition of TNF- α production by nonstimulatory PS-ODN. Intracellular TNF- α was measured by flow-cytometric analysis. RAW264 cells were plated at 2×10^5 cells/well in one mL of medium in 24-well plates overnight. Nonstimulatory PS-ODN NAOS-1 ($0.75 \mu\text{M}$) was added, followed after 30 min by brefeldin A ($5 \mu\text{g/mL}$) to aid intracellular retention of TNF- α , and PO-ODN (AO-1) or PS-ODN (AOS-1) at various concentrations. After a four hour incubation cells were immuno-stained for TNF- α as described (50). Data are expressed as the percentage of total cells expressing TNF- α . The same pattern was obtained in two additional experiments.

face molecules. Induction of the transcription factor Sp1 has also been observed (44). Backbone-specific inhibitory and stimulatory activities of PS-ODN are likely to be owing to their strong interaction with a range of cellular proteins (reviewed in 45–47). For example, apart from strong binding to undefined factors on the cell surface, PS-ODN can bind and inhibit isoforms of protein kinase C (48) and block signaling through growth factor receptors (49).

4. CONCLUSION

Our work, as well as that of others, suggests that PS-ODN have a number of stimulatory and inhibitory activities unrelated to CpG status. PS-ODN do not perfectly mimic native bacterial DNA. This may be an advantage or a disadvantage in a clinical setting, and it is possible that other backbone modifications may give qualitatively different responses worth investigating for therapeutic use. For experimental use, the type of DNA used should depend on the purpose of the study. With a therapeutic application in mind, a PS-ODN

is the obvious choice. If the aim of experiments is to understand the role of DNA in response to infection then the use of PS-ODN is not necessary or appropriate. Furthermore, if the aim is to establish the mechanism of action of natural CpG DNA, then additional activating or inhibitory signals generated by PS-ODN may complicate the analysis. Our recent results show that delayed signaling in response to PS-ODN is sequence-specific. Current work is focusing on defining the sequence elements responsible for delayed signaling.

REFERENCES

1. Krieg, A. M., Yi, A.-K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
2. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.
3. Agrawal, S., Tamsamani, J., Galbraith, W., and Tang, J. (1995) Pharmacokinetics of antisense oligonucleotides. *Clin. Pharmacokinet.* **28**, 7–16.
4. Pisetsky, D. S. and Reich, C. F. (1999) Influence of backbone chemistry on immune activation by synthetic oligonucleotides. *Biochem. Pharmacol.* **58**, 1981–1988.
5. Pisetsky, D. S. (1999) The influence of base sequence on the immunostimulatory properties of DNA. *Immunol. Res.* **19**, 35–46.
6. Zhao, Q., Song, X., Waldschmidt, T., Fisher, E., and Krieg, A. M. (1996) Oligonucleotide uptake in human hematopoietic cells is increased in leukemia and is related to cellular activation. *Blood* **88**, 1788–1795.
7. Branda, R. F., Moore, A. L., Mathews, L., McCormack, J. J. and Zon, G. (1993) Immune stimulation by an antisense oligomer complementary to the *rev* gene of HIV-1. *Biochem. Pharmacol.* **45**, 2037–2043.
8. Krieg, A. M., Matson, S., and Fisher, E. (1996) Oligodeoxynucleotide modifications determine the magnitude of B cell stimulation by CpG motifs. *Antisense Nucleic Acid Drug Dev.* **6**, 133–139.
9. Monteith, D. K., Henry, S. P., Howard, R. B., et al. (1997) Immune stimulation- a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Res.* **12**, 421–432.
10. Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S., and Lipsky, P. E. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
11. Krieg, A. M., Hartmann, G., and Yi, A.-K. (2000) Mechanism of action of CpG DNA. *Curr. Top. Microbiol. Immunol.* **247**, 1–21.
12. Boggs, R. T., McGraw, K., Condon, T., S., F., Villiet, P., Bennett, F. and Monia, B. P. (1997) Characterization and modulation of immune stimulation by modified oligonucleotides. *Antisense Nucl. Acid Drug Dev.* **7**, 461–471.

13. Yi, A.-K., Chace, J. H., Cowdery, J. S., and Krieg, A. M. (1996) IFN- γ promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J. Immunol.* **156**, 558–564.
14. Hartmann, G., Weiner, G. J. and Krieg, A. M. (1999) CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* **96**, 9305–9310.
15. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3049.
16. Kadowaki, N., Antonenko, S., and Liu, Y. J. (2001) Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded rna, respectively, stimulate CD11c(-) type 2 dendritic cell precursors and CD11c(+) dendritic cells to produce type I IFN. *J. Immunol.* **166**, 2291–2295.
17. Lapatschek, M. S., Gilbert, R. L., Wagner, H., and Miethke, T. (1998) Activation of macrophages and B lymphocytes by an oligodeoxynucleotide derived from an acutely pathogenic simian immunodeficiency virus. *Antisense Nucleic Acid Drug Dev.* **8**, 357–370.
18. Hartmann, G. and Krieg, A. M. (2000) Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* **164**, 944–953.
19. Mannon, R. B., Nataraj, C. and Pisetsky, D. S. (2000) Stimulation of thymocyte proliferation by phosphorothioate DNA oligonucleotides. *Cell. Immunol.* **201**, 14–21.
20. Ballas, Z. K., Rasmussen, W. L. and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
21. Lee, S. W., Song, M. K., Baek, K. H., et al. (2000) Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. *J. Immunol.* **165**, 3631–3639.
22. Verthelyi, D., Ishii, K., Gursel, M., Takeshita, F., and Klinman, D. (2001) Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J. Immunol.* **166**, 2372–2377.
23. Pisetsky, D. S. and Reich, C. F. (2000) Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. *Clin. Immunol.* **96**, 198–204.
24. Lang, R., Hultner, L., Lipford, G. B., Wagner, H., and Heeg, K. (1999) Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. *Eur. J. Immunol.* **29**, 3496–3506.
25. Stacey, K. J., Sweet, M., and Hume, D. A. (1996) Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116–2122.
26. Sweet, M. J., Stacey, K. J., Kakuda, D. K., Markovich, D. and Hume, D. A. (1998) IFN- γ primes macrophage responses to bacterial DNA. *J. Interferon Cytokine Res.* **18**, 263–271.
27. Sester, D. P., Naik, S., Beasley, S. J., Hume, D. A., and Stacey, K. J. (2000) Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. *J. Immunol.* **165**, 4165–4173.

28. Schindler, U. and Baichwal, V. R. (1994) Three NF- κ B binding sites in the human E-selectin gene required for maximal tumor necrosis factor α -induced expression. *Mol. Cell Biol.* **14**, 5820–5831.
29. Sester, D. P., Beasley, S. J., Sweet, M. J., et al. (1999) Bacterial/CpG DNA Down-Modulates Colony Stimulating Factor-1 Receptor Surface Expression on Murine Bone Marrow-Derived Macrophages with Concomitant Growth Arrest and Factor-Independent Survival. *J. Immunol.* **163**, 6541–6550.
30. Häcker, H., Mischak, H., Hacker, G., et al. (1999) Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. *EMBO J.* **18**, 6973–6982.
31. Yi, A. K. and Krieg, A. M. (1998) Cutting edge: Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493–4497.
32. Takeshita, F., Ishii, K. J., Ueda, A., Ishigatsubo, Y., and Klinman, D. M. (2000) Positive and negative regulatory elements contribute to CpG oligonucleotide-mediated regulation of human IL-6 gene expression. *Eur. J. Immunol.* **30**, 108–116.
33. Takeshita, F. and Klinman, D. M. (2000) CpG ODN-mediated regulation of IL-12 p40 transcription. *Eur. J. Immunol.* **30**, 1967–1976.
34. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
35. Takakura, Y., Oka, Y., and Hashida, M. (1998) Cellular uptake properties of oligonucleotides in LLC-PK₁ renal epithelial cells. *Antisense Nucl. Acid Drug Dev.* **8**, 67–73.
36. Zhao, Q., Matson, S., Herrera, C. J., et al. (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res. Dev.* **3**, 53–56.
37. Nakai, D., Seita, T., Terasaki, T., et al. (1996) Cellular uptake mechanism for oligonucleotides: Involvement of endocytosis in the uptake of phosphodiester oligonucleotides by a human colorectal adenocarcinoma cell line, HCT-15. *J. Pharmacol. Exp. Ther.* **278**, 1362–1372.
38. Tonkinson, J. L. and Stein, C. A. (1994) Patterns of intracellular compartmentalization, trafficking and acidification of 5'-fluorescein labeled phosphodiester and phosphorothioate oligodeoxynucleotides in HL60 cells. *Nucl. Acids Res.* **22**, 4268–4275.
39. Beltinger, C., Saragovi, H. U., Smith, R. M., et al. (1995) Binding, uptake, and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides. *J. Clin. Invest.* **95**, 1814–1823.
40. Bennett, R. M. (1993) As nature intended? The uptake of DNA and oligonucleotides by eukaryotic cells. *Antisense Res. Dev.* **3**, 235–241.
41. Benimetskaya, L., Loike, J. D., Khaled, Z., et al. (1997) Mac-1 (CD11b/CD18) is an oligodeoxynucleotide-binding protein. *Nature Med.* **3**, 414–420.
42. Häcker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.

43. Hartmann, G., Krug, A., Waller-Fontaine, K., and Endres, S. (1996) Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: dependence on phosphorothioate modification and reversal by heparin. *Mol. Med.* **2**, 429-438.
44. Perez, J. R., Li, Y., Stein, C. A., Majumder, S., van Oorschot, A., and Narayanan, R. (1994) Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. *Proc. Natl Acad. Sci. USA* **91**, 5957-5961.
45. Neckers, L. M. and Iyer, K. (1997) Non-antisense effects of antisense oligonucleotides, in *Antisense oligodeoxynucleotides and antisense RNA* (Weiss, B., ed.), CRC Press, Boca Raton, pp. 80-89.
46. Stein, C. A. (1996) Phosphorothioate antisense oligodeoxynucleotides: questions of specificity. *Trends Biotechnol.* **14**, 147-149.
47. Eckstein, F. (2000) Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* **10**, 117-121.
48. Khaled, Z., Rideout, D., O'Driscoll, K. R., et al. (1995) Effects of suramin-related and other clinically therapeutic polyanions on protein kinase C activity. *Clin. Cancer Res.* **1**, 113-122.
49. Rockwell, P., O'Connor, W. J., King, K., Goldstein, N. I., Zhang, L. M., and Stein, C. A. (1997) Cell-surface perturbations of the epidermal growth factor and vascular endothelial growth factor receptors by phosphorothioate oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **94**, 6523-6528.
50. Underhill, D. M., Ozinsky, A., Hajjar, A. M., et al. (1999) The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* **401**, 811-815.

PART III

CELL ACTIVATION

Activation of NK Cell By Immunostimulatory Oligo-DNA in Mouse and Human

Saburo Yamamoto, Toshiko Yamamoto, Tetsuro
Kataoka, Sumiko Iho, and Tohru Tokunaga

1. MODE OF ACTION OF MY-1

It was reported that a purified DNA-rich fraction, MY-1, extracted from *Mycobacterium bovis* Bacille Calmette Guérin BCG, exhibits a strong anti-tumor activity against various syngeneic mouse and guinea pig tumors by activating the host innate immune response (1,2). This fraction showed no direct cytotoxicity in vitro against these tumors. An intraperitoneal injection of MY-1 rendered mouse peritoneal cells strongly cytotoxic to YAC-1 cells in vitro, but not to P815 cells. This activity was destroyed by treatment with antiasialo-GM1 antiserum, suggesting that the cells are natural killer (NK) cells. When mice were pretreated with antiasialo-GM1, MY-1 could not render the peritoneal cells cytotoxic. Antitumor activity of MY-1 was also abolished if the animals were pretreated with asialo-GM1 antiserum suggesting that the activity can be ascribed mainly to activated NK cells (3). MY-1 also augmented NK cell activity of mouse spleen cells in vitro, and produced factors which showed antiviral activity as interferon (IFN)- α/β and rendered macrophages cytotoxic towards tumor cells as IFN- γ (4). The activity reached the highest level after 6 h of incubation. A significant elevation of NK activity was seen after 1 h, while neither IFN α/β nor IFN γ was observed within 3 h. These cellular responses were induced by the RNase-digested MY-1 almost the same as those of MY-1, but no activity was seen in the DNase-digested MY-1, indicating that DNA containing MY-1

was essential for the responses. It appears that DNA from BCG stimulated mouse spleen cells in vitro results in augmentation of NK cells.

1.1. Particular Base Sequences in Bacterial-DNA is Required for NK Augmentation

The DNA molecules comprising MY-1 showed a wide range of molecular sizes from 10 to 350 bases, with the peak position estimated to be 45 bases. Thirteen kinds of 45-mer single-stranded oligoDNA each having a sequence randomly chosen from cDNA sequence encoding BCG proteins were synthesized, and assayed for their ability to augment mouse NK activity in vitro. Interestingly only 6 of the 13 oligoDNAs showed strong activity to augment mouse NK activity, whereas the others did not. The activity to induce IFN paralleled the activity to augment NK cell, suggesting that some unique sequences are required for expressing immunostimulatory activity. We focused on one particular oligoDNA with 45-mer, A4, to clarify the sequence responsible for the activity. The ability of two portions of A4 spanning 30 bases to augment NK activity was examined, one was active (A4a), but the other was inactive (A4b). Since A4a has a palindrome sequence (GACGTC), whereas A4b does not, the possible importance of the palindromic sequence was suggested for the active oligoDNAs (5).

A variety of 30-mer single-stranded oligoDNAs were synthesized, and the NK-stimulatory activity was examined. The activity of oligoDNAs with GACGTC sequence was stronger than that of oligoDNAs without GACGTC, which suggested the necessity of the palindromic sequence for the activity. When the GACGTC palindrome was introduced into the sequence of inactive oligoDNAs, the modified oligoDNAs acquired the NK-stimulatory activity, irrespective of the position in the sequence at which the palindrome was introduced. OligoDNAs lost their activity after nucleotide exchange within, but not outside the active palindromic sequence (6).

Based on the finding that 30-mer single-stranded oligoDNAs with particular hexamer palindromic sequences could enhance NK activity, a study was performed to clarify the entire relationship between the activity and the sequence of 30-mer oligoDNAs. The results indicated that the activity depended critically on the presence of particular palindromic sequences including the 5'-CpG-3' motif(s). The size and the number of palindromes as well as the extra-palindromic sequences also influenced activity. All the active oligoDNAs included a palindromic sequence, such as GACGTC, AGCGCT, and AACGTT, whereas the inactive ones did not. When a portion of an inactive oligoDNA was substituted with a palindrome from an active oligoDNA, the oligoDNA acquired the ability to enhance NK activity. In contrast, a sequence substitution with an ACCGGT palindrome did

not give rise to activity. Furthermore, the active oligoDNAs lost activity after an exchange or a deletion of bases within, but not outside, the hexamer palindromic sequence. Taken together, these findings indicate that some, but not all, of the hexamer palindromic sequences are essential for the activity of oligoDNAs. Extra-palindromic sequences have also appeared to be necessary, because trimming an active 45-mer oligoDNA molecule to 15-mer, whereas keeping the palindromic sequence intact, resulted in decreased activity. The palindromic sequence (5'-GACGTC-3') of an active oligoDNA, A4a, the sequence of which is 5'-accgatGACGTCgccggtgacggcaccacg-3', was replaced with each of the 63 theoretically possible hexamer palindromic sequences. More than 100% relative activity was observed in the eight oligoDNAs with one of the following palindromic sequences: AACGTT, AGCGTC, ATCGAT, CGATCG, CGTACG, CGCGCG, GCGCGC, and TCGCGA. All the potent palindromes included one or more 5'-CpG-3' motif(s). In contrast, palindromes composed entirely of adenosine (A) and thymine (T) and those with a sequence of either Pu-Pu-Pu-Py-Py-Py or Py-Py-Py-Pu-Pu-Pu (Pu, purine; Py, pyrimidine) were generally unfavorable for activity (7).

1.2. Antitumor Activity of oligoDNAs Correlates with NK Augmenting Activity

Thirteen kinds of 45-mer or 30-mer oligoDNAs with sequences randomly selected from the cDNA encoding BCG proteins were assayed for their antitumor activity in murine tumor system (Table 1). Of the 13 single-stranded oligoDNAs that contained one or more hexameric palindromic sequences, six showed strong antitumor activity whereas the others without palindromic structure did not. Repeated intralesional injection of 100 µcg of the six oligoDNAs caused regression of the established tumor but the others were ineffective. When IMC tumor cells were mixed with A4 or A4a oligoDNA, including GACGTC palindromic sequence, the tumor growth was markedly suppressed, whereas the other oligoDNA without palindrome did not significantly effect the tumor growth. Thus, the antitumor activity of the oligoDNAs correlated well with the NK augmenting activity (Fig. 1). Furthermore, these activities also correlated with the IFN inducing activity (8).

1.3. Ability of oligoDNAs with Palindromes to Augment NK Activity is Associated with Base Length

Ten kinds of 12- to 30-mer nucleotides were examined to clarify the required minimal size of the oligoDNAs. To prepare the various lengths of oligoDNAs possessing AACGTT palindromic sequence, the extra-palindromic sequence of a potent oligoDNA, AAC-30, was trimmed stepwise. Trimming two nucleotides from the 3' end of the AAC-30 oligoDNA resulted in

Table 1
The Sequences of OligDNAs Used

Name	Base Sequence
A1	AACGAG GGGCAT GACCCG GTGCGG GGCTTC TTGCAC TCGGCA TAG
A2	AAAAGA AGTGGG GTGCCC CCACGA TCACCA ACGATG GTGTGT CCA
A3	TCCATC GCCAAG GAGATC GAGCTG GAGGAT CCGTAC GAGAAG ATC
A4	ACCGAT GACGTC GCCGGT GACGGC ACCACG ACGGCC ACCGTG CTG
A4a	ACCGAT GACGTC GCCGGT GACGGC ACCACG
A4b	GGT GACGGC ACCACG ACGGCC ACCGTG CTG
A5	TATGCG GTTCGA CAAGGG CTACAT CTCGGG GTACTT CGTGAC CGA
A6	ACGAGA CCACCA TCGTCG AGGGCG CCGGTG ACACCG ACGCCA TCG
A7	GCCGAG AAGGTG CGCAAC CTGCCG GCTGGC CACGGA CTGAAC GCT
A8	ACCGAG AACAGC CACGCA GTCGTG TAGGCA ACCTTT GGCCGC TGT
M1	GGCGAT CTGGTG GGCCCG GGCTGC GCGGAA TACGCG GCAGGC AAT
M3	ACGCCG ACGTCG TCTGTG GTGGGG TGTCTA CCGCCA ACGCGA CGG
α	CGACTA CAACGG CTGGGA TATCAA CACCCC GGC GTT CGAGTG GTA

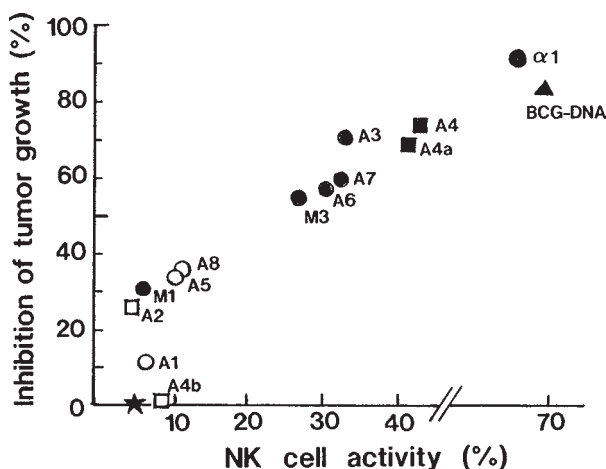


Fig 1. Correlation between antitumor activity and NK augmenting activity induced by the oligoDNAs. Closed circles and squares (● & ■) represent oligoDNA with palindromic sequences and open circles and squares (○ & □) represent those without a palindromic sequences. For comparison, values obtained by cultivation with DNA fraction extracted from BCG (▲, closed triangle) and with medium alone (★, closed star) are presented.

AAC-28. By repeating this procedure, AAC-26, AAC-24, AAC-22, AAC-20, AAC-18, AAC-16, AAC-14, and AAC-12 were selected. In these cases, only the extra-palindromic sequences were trimmed and the hexamer palindrome of AACGTT was maintained. The activity of these oligoDNAs in augmenting NK activity was examined by co-culture of mouse spleen cells with either of these oligoDNAs. Immunostimulatory activity of oligoDNAs with 18 or more bases was proportional to the base length, with a maximum at 22–30 bases. On the other hand, the oligoDNAs with 16 or fewer bases were not active even if they possessed certain palindromic sequences. These results indicate that for oligoDNAs with certain palindromes to have immunostimulatory activity they require at least 18 bases (9).

1.4. Lipofection of An oligoDNA with a Palindromic Sequence to Mouse Spleen Cells Enhances NK Activity

A synthetic 22-mer oligoDNA having an AACGTT palindrome, AAC-22, the sequence of which is 5'-accgatAACGTTgccggtgacg-3', augmented or so the NK activity and induced IFN production after co-cultured with mouse spleen cells, whereas its analog, ACC-22, having an ACCGGT pal-

indrome instead of AACGTT did not. The binding of AAC-22 to spleen cells did not differ from that of ACC-22. Lipofection of AAC-22 to splenocytes remarkably enhanced IFN production and NK cell activity, whereas that of ACC-22 caused little enhancement. These results strongly suggest that the prerequisite for IFN production is not the binding of AAC-22 to the cell surface receptors, but its penetration into the spleen cells (10).

A hexamer palindromic oligoDNA (5'-AACGTT-3') induced IFN from cultured mouse spleen cells when added with cationic liposomes. Accordingly, 32 kinds of hexamer palindromic oligoDNAs were tested for their ability to induce IFN in the presence of cationic liposomes. The result shows that oligoDNAs with NACGTN and NTCGAN sequences exhibited the strongest activity. ACGCGT and TCGCGA also possessed moderate but significant activity. In contrast, palindromes without CG motif(s) were devoid of the activity. No hexamer oligoDNAs showed the activity when liposomes were absent. A complete palindromic sequence was essential as any single base substitution resulted in diminished activity. Among a variety of palindromic oligoDNAs of different sizes with an ACGT sequence at the center, the tetramer oligoDNA was without activity, whereas the activity of hexamer and longer oligoDNAs was almost equally high. These results strongly suggest that the minimal essential structure required for IFN induction is the hexamer palindromic sequence with CpG motif(s) (11).

1.5. OligoDNAs with CpG Motif Stimulate Human PBL In Vitro

The ability of single-stranded 30-mer oligoDNAs with three different kinds of hexamer palindromic sequences to stimulate IFN production by human PBL was studied. When PBL were cultured with oligoDNAs with an AACGTT or GACGTC palindrome, IFN activity was detected by bioassay in the culture fluid after 8 h, and the amount of IFN reached the maximum after 18 h. IFN- α was predominantly produced, and small amount of IFN- β and IFN- γ were also found. OligoDNAs with the ACCGGT palindrome had no effect (12).

We examined the effect of oligoDNAs on highly purified human NK and T cells (13). MY-1 or synthetic oligoDNAs induced NK cells to produce IFN- γ with increased CD69 expression, and the autocrine IFN- γ enhanced their cytotoxicity. The response of NK cells to oligoDNAs was enhanced when the cells were activated with IL-2, IL-12 or anti-CD16 antibody. T cells did not produce IFN- γ in response to oligoDNAs but did respond independently of IL-2 when they were stimulated with anti-CD3 antibody. In the action of oligoDNAs, the palindrome sequence containing unmethylated CG motif(s) appeared to play an important role in IFN- γ producing ability of NK cells. The changes of base composition inside or outside the palindrome

sequence altered its activity. The homo-oligoG-flanked GACGATCGTC was the most potent IFN- γ inducer for NK cells and T cells. The CG-palindrome was also important for IFN- γ production in activated NK and T cells, although certain nonpalindromes also acted on them. Among the sequences tested, cell activation- or cell lineage-specific sequences were found; i.e., palindrome ACCGGT and nonpalindromic AACGAT were favored by activated NK cells but not by nonactivated NK or activated T cells. These results indicate that oligoDNAs containing CG palindrome act directly on human NK and activated T cells to induce IFN- γ production. To clarify the cell type targeted by immunostimulatory oligoDNAs to produce IFN- γ , we separated large granular lymphocytes (LGL) and T cells from nonadherent cells (NAC). When these two cell fractions were cultured for 24 h, only the LGL fraction produced IFN- γ in the presence of MY-1. NAC, which contained 20–30% NK and 70–80% T cells, produced IFN- γ in response to MY-1 when the cell density was increased to 4×10^6 cells/mL, whereas the purified T-cell fraction did not produce IFN- γ even when cultured at 1×10^7 cells/mL or for longer periods. We purified CD56+ cells from the LGL fraction and found that they were responsive to MY-1. NK cells produced IFN- γ in response to MY-1. The doses of MY-1 necessary to induce the maximum amounts of IFN- γ were between 12.5–50 μ g/mL in NK cell culture. IFN- γ production in the culture with MY-1 was first observed at 18 h and increased thereafter. The amount of IFN- γ produced without MY-1 at 24 h of culture was below 4 pg/mL and did not increase. These results show that NK cells are responsive in terms of IFN- γ production. To prove that the MY-1 induced IFN- γ production is due to a direct action on NK cells, the effect of monoclonal antibody against IL-12 or TNF- α on the IFN- γ production of NK cells in the stimulation of oligoDNAs was examined. Neither anti-IL-12 nor anti-TNF- α monoclonal antibody influenced the IFN- γ production by NK cells cultured with or without oligoDNA. The combined addition of monoclonal antibodies against IL-12 and TNF- α also did not inhibit the production of IFN- γ . In addition, monoclonal antibody to IL-18, IL-15 or IFN- α did not alter the level of IFN- γ production induced by the oligoDNAs (13).

2. DISCUSSION

We pointed out in 1992 that hexamer palindromic sequence having CpG-motif(s) is essential for NK augmentation and IFN production, with some exceptions (7). Krieg et al., also noted the importance of CpG motif as the basic structure required for B-cell stimulation, and stated that the CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines is optimal (14). Ballas et al., examined the necessity of palindromic sequences for NK

activation and observed that the presence of hexamer palindrome was irrelevant; for instance, GTCGTT and GACGTT were active, and GACGTC was inactive (15). They concluded that unmethylated CpG motif, but not palindromic sequence, was definitely required, and two flanking bases at the 5' and 3' ends were a stringent requirement. Boggs et al., tested various sequences of oligoDNAs, and showed that the CpG motif was stimulatory for NK cells only in specific sequence contexts (16). Monteith et al., tested various oligoDNA sequences for inducing splenomegaly and B-cell stimulation; oligoDNAs including a 5'-AACGTT-3' palindrome were the most effective (17). The general formula, Pu-Pu-CpG-Py-Py, proposed by Krieg [14] and Klinman (18), has also many exceptions, although the formula has been widely used by many investigators. Therefore we think at present that either the "immunostimulatory sequences (ISS) of DNA" (19–21) or the "CpG dinucleotides in particular base contexts" (22) is appropriate to use for indicating immunostimulatory sequences.

REFERENCES

1. Tokunaga, T., Yamamoto, H., Shimada, S., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–962.
2. Shimada, S., Yano, O., Inoue, H., et al. (1985) Antitumor activity of the DNA fraction from *Mycobacterium bovis* BCG. II. Effects on various syngeneic mouse tumors. *J. Natl. Cancer Inst.* **74**, 681–688.
3. Shimada, S., Yano, O., and Tokunaga, T. (1986) *In vivo* augmentation of natural killer cell activity with a deoxyribonucleic acid fraction of BCG. *Jpn. J. Cancer Res.* **77**, 808–816.
4. Yamamoto, S., Kuramoto, E., Shimada, S., and Tokunaga, T. (1988) *In vitro* augmentation of natural killer cell activity and production of interferon- α/β and - γ with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **79**, 866–873.
5. Tokunaga, T., Yano, O., Kuramoto, E., et al. (1992) Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. *Microbiol. Immunol.* **36**, 55–66.
6. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J Immunol.* **148**, 4072–4076.
7. Kuramoto, E., Yano, O., Kimura, Y., et al. (1992) Oligonucleotide Sequences Required for Natural killer Cell Activation. *Jpn. J. Cancer Res.* **83**, 1128–1131.
8. Kataoka, T., Yamamoto, S., Yamamoto, T., et al. (1992) Antitumor Activity of Synthetic Oligonucleotides with Sequences from cDNA Encoding Proteins of *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **83**, 244–247.

9. Yamamoto, T., Yamamoto, S., Kataoka, T., and Tokunaga, T. (1994) Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. *Antisense Res. Develop.* **4**, 119–122.
10. Yamamoto, T., Yamamoto, S., Kataoka, T., and Tokunaga, T. (1994) Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. *Microbiol. Immunol.* **38**, 831–836.
11. Sonehara, K., Saito, H., Kuramoto, E., Yamamoto, S., Yamamoto, T. and Tokunaga, T. (1996) Hexamer palindromic oligonucleotides with 5'-CG-3' motif(s) induce production of interferon. *J. IFN Cytokine Res.* **16**, 799–803.
12. Yamamoto, T., Yamamoto, S., Kataoka, T., Komuro, K., Kohase, M., and Tokunaga, T. (1994) Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes *in vitro* *Jpn. J. Cancer Res.* **85**, 775–779.
13. Iho, S., Yamamoto, T., Takahashi, T., and Yamamoto, S. (1999) Oligodeoxynucleotides containing palindrome sequences with internal 5'-CpG-3' act directly on human NK and activated T cells to induce IFN- γ production *in vitro*. *J. Immunol.* **163**, 3642–3652.
14. Krieg, A. M., Yi, A.-K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
15. Ballas, Z. K., Rasmussen W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
16. Boggs, R. L., McGraw, K., Condon, T., et al. (1997) Characterization and modulation of immune stimulation by modified oligonucleotides. *Antisense Nucleic Acid Drug Develop.* **7**, 461–471.
17. Monteith, D. K., Henry, S. P., Howard, R. B., et al. (1997) Immune stimulation—a class effect of phosphorothioate oligonucleotides in rodents. *Anti-Cancer Drug Design* **12**, 421–432.
18. Klinman, D. M., Yi, A.-K., Beaucaga, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2833.
19. Roman, M., Orozco, E. M., Goodman, J. S., Nguyen, M. D., Sato, Y., and Raz, E. (1997) Immunostimulatory DNA sequences function as T helper-1 promoting adjuvants. *Nature Med.* **3**, 849–854.
20. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
21. Tighe, H., Corr, M., Roman, M., and Ratz, E. (1998) Gene vaccination: plasmid DNA is more than just a blueprint. *Immunology Today* **19**, 89–97.
22. Yi, A.-K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J., and Krieg, A. M. (1998) CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* **160**, 4755–4761.

Regulation of Antigen Presenting Cell Function by CpG DNA

David Askew, Rose S. Chu, and Clifford V. Harding

1. INTRODUCTION

The immune system responds to certain microbial components or pathogen-associated molecular patterns (PAMPs) (1,2), including lipopolysaccharide (LPS), peptidoglycan, bacterial lipopeptides, lipoteichoic acid, microbial polysaccharides (e.g., mannans) and CpG DNA motifs present in bacterial DNA (3). The immune system uses pattern recognition receptors like the toll-like receptors (TLRs) to recognize these bacterial components, and transduce signals that modify the function of leukocytes. For example, TLR4 is involved in responses to LPS, TLR2 mediates responses to bacterial lipopeptides, and CpG DNA is recognized by TLR9 (4). MyD88 appears to be a common immediate downstream element in TLR signaling and is involved in recognition of CpG DNA (5).

Recognition of bacterial DNA is owing to the presence of unmethylated CpG motifs, e.g., “purine-purine-unmethylated C-G-pyrimidine-pyrimidine,” although a more inclusive motif may be “not C, unmethylated C, G, not G” (3,6–8). Other studies have investigated immunostimulatory sequences (ISS) that have effects similar to those of CpG motifs (9), and our discussion of the effects of these DNA preparations does not generally distinguish CpG motifs and immunostimulatory sequences. In mammalian DNA, CpG motifs are suppressed in frequency and also inactivated by cytosine methylation. Synthetic oligodeoxynucleotides (ODN) or plasmid DNA preparations that contain CpG motifs mimic the effects of bacterial DNA in modulation of immune responses.

CpG ODN induce dendritic cells, macrophages, B cells, and NK cells to express proinflammatory cytokines, e.g., IL-12, TNF- α and interferon- γ (10–16). In addition, CpG ODN increase expression of class I MHC (MHC-I)

molecules, class II MHC (MHC-II) molecules, CD40, CD80, and CD86 in dendritic cells (11,17–20), although not all monocyte/macrophage/dendritic cell populations respond similarly (20,21).

Administration of CpG ODN or related DNA preparations with protein antigen *in vivo* enhances T cell responses and promotes differentiation of T helper 1 (Th1) responses. These Th1 responses are characterized by increased production of interferon- γ and decreased production of IL-5 upon antigenic recall stimulation *in vitro*, and production of antigen-specific IgG2a, a Th1-associated antibody isotype (9,22). CpG ODN also promote development of cytotoxic T cell (CTL) responses (23,24). Overall, these DNA preparations have potent adjuvant activities in a number of vaccine applications (9,22,23,25–33), promote protective Th1 responses, e.g. in the setting of leishmaniasis (34,35) or schistosomiasis (36), and decrease deleterious Th2-associated effects, e.g., in the setting of allergy and asthma (16,37,38).

Several mechanisms may contribute to the adjuvant activity of CpG ODN. Enhancement of T-cell responses may occur through modulation of antigen processing, antigen presentation, cytokine responses or other mechanisms. Enhanced antigen processing would increase the number of peptide:MHC complexes that are presented to T cells by macrophages, dendritic cells or B cells. In addition, enhanced expression of costimulator molecules could increase antigen presentation. Our studies have demonstrated that CpG ODN enhance both antigen processing and costimulator expression in dendritic cells, although somewhat different results were obtained with macrophages.

2. EFFECTS OF CpG ODN ON ANTIGEN PROCESSING AND PRESENTATION

2.1. *Effects of CpG ODN on Macrophage Antigen Processing*

To determine the effect CpG ODN on macrophage antigen processing and presentation, concanavalin A-elicited peritoneal macrophages were incubated overnight (18–24 h) in the presence or absence of CpG ODN, washed and incubated with protein antigen for 1–2 h (39). The cells were then washed and fixed to prevent further antigen processing. T hybridoma cells were added to detect peptide:MHC-II complexes expressed by macrophages. CpG ODN suppressed antigen processing and formation of peptide:MHC-II complexes from two model antigens, bovine ribonuclease A (RNase) and hen egg lysozyme (HEL) (Fig. 1). Antigen processing was not inhibited by prior incubation with non-CpG ODN (39). Inhibition was seen after overnight incubation with CpG ODN (18–24 h), but not with shorter incubations of a few hours. CpG ODN did not enhance macrophage

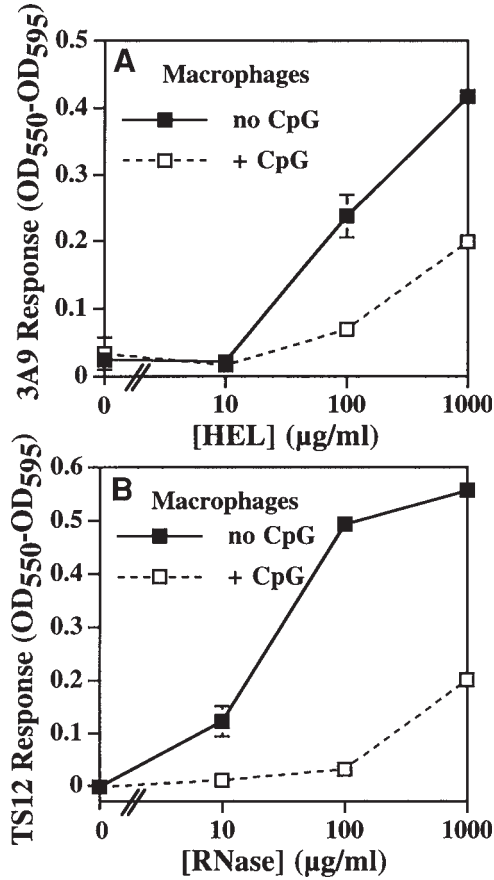


Fig. 1. Treatment of macrophages with CpG ODN for 18–24 h inhibits antigen processing. Concanavalin A-elicited peritoneal macrophages from CBA/J mice were incubated overnight with or without CpG ODN. The cells were washed, incubated for 1 h with HEL (panel A) or RNase (panel B), fixed and incubated with T-cell hybridoma cells (3A9 cells were used to detect HEL₄₈₋₆₁:I-A^k complexes and TS12 cells were used to assess presentation of RNase₄₂₋₅₆:I-A^k complexes). T cell response was assessed by IL-2 secretion, which was measured using a colorimetric bioassay with CTLL-2 cells. Each data point represents the mean of triplicate wells ($\pm$ SD. If error bars are not visible, they are smaller than the symbols. Adapted and reproduced with permission from Chu et al. (39), (copyright 1999, The American Society of Immunologists).

antigen processing at any time point, even when macrophages were pulsed with antigen prior to CpG ODN exposure. When macrophages were treated with or without CpG ODN and fixed, subsequent presentation of exogenous

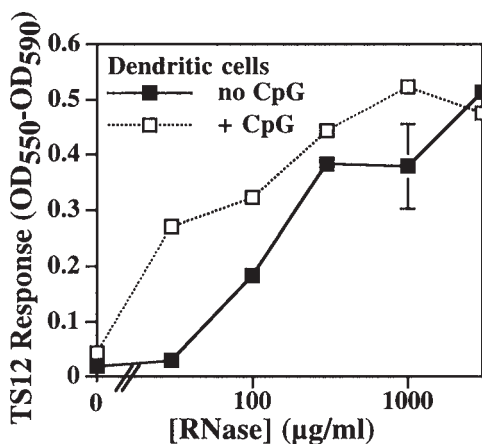


Fig. 2. CpG ODN enhance dendritic cell antigen processing within 24–48 h. Dendritic cells were cultured with RNase in the presence or absence of CpG ODN for 24 h, washed and incubated for an additional 24 h prior to fixation. Presentation of RNase₄₂₋₅₆:I-A^k complexes was detected as in Fig. 1. Each data point represents the mean of triplicate wells ($\pm$ SD. If error bars are not visible, they are smaller than the symbols. Adapted and reproduced with permission from Askew et al. (19), (copyright 2000, The American Society of Immunologists).

peptide was suppressed by CpG ODN, suggesting that CpG ODN decreased expression of peptide-receptive MHC-II molecules on macrophages. To measure the effect of CpG ODN on MHC-II expression, macrophages were incubated with CpG ODN or non-CpG ODN overnight, and expression of MHC-II was measured by flow cytometry. CpG ODN decreased surface expression of MHC-II (39). After similar treatment with CpG ODN, macrophage expression of mRNA for MHC-II was decreased (to 24% of control for I-A^k) (39). Endocytosis and degradation of antigen were not affected by treatment with CpG ODN. Thus, CpG ODN suppress macrophage antigen processing primarily by downregulation of MHC-II synthesis.

2.2. Effects of CpG ODN on Dendritic Cell Antigen Processing

Although CpG ODN did not enhance antigen processing by macrophages, a different pattern has emerged in studies of dendritic cells from our laboratory (19) and others (11,17,20). In our studies, dendritic cells were isolated from day 6 bone marrow cultures, incubated with antigen +/- CpG ODN for 24 h, washed, and fixed after an additional 24 h. CpG ODN enhanced processing of both RNase (Fig. 2) and HEL (19) for presentation to T cells. Treatment with CpG ODN increased the stability of peptide:MHC-II complexes expressed by dendritic cells (19). Thus, expo-

sure of dendritic cells to CpG ODN prolonged the expression of peptide:MHC-II complexes formed during the incubation with antigen and CpG ODN, enhancing their presentation to T cells.

CpG ODN induce maturation of dendritic cells (11,17,19,20), similar to LPS and other PAMPs (40,41). Since maturation of dendritic cells is associated with decreased antigen processing, we examined antigen processing in dendritic cells after a 48-h exposure to CpG ODN (19). In addition, we examined the effects of CpG-induced maturation on two different antigen processing mechanisms. HEL is processed to HEL₄₈₋₆₁:I-A^k complexes via a late endocytic mechanism that uses nascent MHC-II, whereas processing of RNase to RNase₄₂₋₅₆:I-A^k complexes occurs via an early endosomal mechanism that uses recycling MHC-II (42). To test the roles of nascent vs recycling MHC-II molecules in antigen processing, brefeldin A was used to block delivery of nascent MHC-II from the endoplasmic reticulum and Golgi complex. Processing of HEL to HEL₄₈₋₆₁:I-A^k was completely inhibited by both CpG ODN and brefeldin A (Fig. 3), confirming that the late endocytic processing of this epitope requires nascent MHC-II and establishing that this processing mechanism was shut down by CpG ODN. Consistent with these functional studies, real-time quantitative PCR showed that mRNA for MHC-II was decreased by CpG-induced maturation of dendritic cells. In contrast, brefeldin A caused only a partial inhibition of RNase processing to produce RNase₄₂₋₅₆:I-A^k complexes, confirming the ability of early endosomal processing of this epitope to utilize recycling MHC-II. CpG ODN inhibited RNase processing to a greater degree, but some residual early endosomal processing activity remained even after CpG-induced maturation of dendritic cells. The residual early endosomal antigen processing activity in mature dendritic cells was completely resistant to brefeldin A, indicating that this component used recycling MHC-II.

Changes in antigen processing induced by CpG ODN are explained by both altered synthesis of MHC-II and altered endocytosis of antigen. During the first day of exposure, CpG ODN increased endocytosis of FITC-dextran by 25% (19). This effect may promote increased formation peptide:MHC-II complexes (in addition to the enhancement of stability of these complexes). However, exposure of dendritic cells to CpG ODN for two days decreased subsequent endocytosis of FITC-dextran by 37%. Thus, CpG-induced maturation of dendritic cells results in a transient increase in endocytosis followed by a long-term decrease in endocytosis.

3. DISCUSSION

CpG ODN are effective adjuvants for protein antigens and augment antigen-specific antibody responses and T-cell responses, including Th1 (CD4)

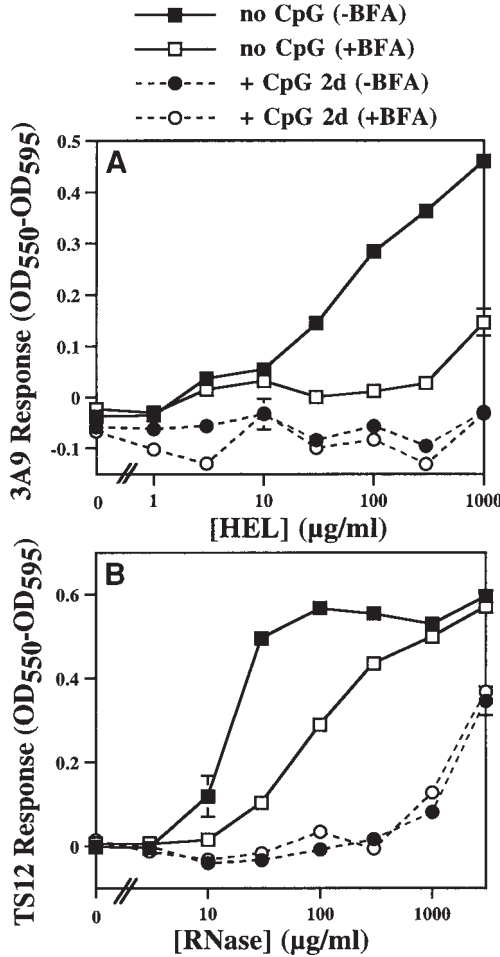


Fig. 3. Long-term effects of CpG ODN include downregulation of antigen processing by dendritic cells. Dendritic cells were cultured for 2 d with or without CpG ODN, incubated for 3 h with or without BFA, incubated for 3 h with antigen in the continued presence or absence of BFA and then fixed. Antigen presentation was determined as in Fig. 1. Each data point represents the mean of triplicate wells ($\pm$ SD). If error bars are not visible, they are smaller than the symbols. Adapted and reproduced with permission from Askew et al. (19), (copyright 2000, The American Society of Immunologists).

and CTL (CD8) responses. Modulation of antigen processing may contribute to the adjuvant function of CpG ODN. We have shown that CpG ODN cause no short-term effect and a long-term inhibition in macrophage antigen

processing (primarily due to decreased MHC-II synthesis). Despite the long-term decrease in macrophage antigen processing, macrophages can still process antigens during the hours immediately after exposure to a vaccine or microbial antigen and thereby stimulate T cells. In addition, macrophages contribute to the adjuvant effects of CpG ODN through the production of proinflammatory cytokines such as IL-12.

Our results also show that CpG ODN affect antigen processing differently in macrophages and dendritic cells. Dendritic cells exhibit a short-term enhancement of antigen processing after exposure to CpG ODN. Contributing mechanisms include an increase in endocytosis and antigen uptake, and an increase in the stability of peptide:MHC-II complexes. At the same time, expression of costimulatory molecules (CD40, CD80, and CD86) is increased. These changes promote enhanced presentation of antigens encountered in the context of the vaccine adjuvant or microbe.

Dendritic cell antigen processing is only transiently enhanced by CpG ODN, and the long-term effects of CpG-induced maturation of dendritic cells include decreased antigen processing. Late endocytic antigen processing mechanisms that require nascent MHC-II are completely inhibited (consistent with inhibition of MHC-II synthesis), whereas early endosomal antigen processing that uses recycling MHC-II is substantially reduced, but not eliminated. In addition to the decrease in MHC-II synthesis, a decrease in endocytosis contributes to the long-term loss of antigen processing function (including mechanisms that use recycling MHC-II). Hypothetically, decreased endocytosis may lower the rate of MHC-II endocytosis, possibly decreasing downregulation of peptide: MHC-II complexes and contributing to enhancement of their stability of peptide: MHC-II.

The data on dendritic cells from our lab and others support a model that we term the “freeze-frame hypothesis.” Antigen processing results in the presentation of a repertoire of peptide: MHC-II complexes that changes with time, as some complexes are lost and replaced by new complexes. Exposure to CpG DNA or other PAMPs in the context of a vaccine adjuvant or microbial infection results in a transient enhancement of antigen processing and production of a cohort of peptide:MHC-II complexes that is enriched in peptides derived from the vaccine or microbe. Antigen processing is then decreased, but the stability of previously formed complexes is increased, prolonging the presentation of these complexes, including those containing microbial or vaccine antigen peptides. We liken the process to a movie that shows an evolving repertoire of complexes available for antigen presentation. Recognition of CpG DNA (19) or other PAMPs (40,41) signals the immune system to freeze attention on one frame of this movie, i.e., the set of

complexes generated near the time of exposure to the PAMP, providing sustained examination of this set of antigens for stimulation of T-cell responses.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI35726, AI44794 and AI47255 to C. V. H. David Askew was supported in part by NIH T32-HL07889.

REFERENCES

1. Medzhitov, R. and Janeway, C., Jr. (2000) Innate immunity. *N. Engl. J. Med.* **343**, 338–344.
2. Aderem, A. and Ulevitch, R. J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406**, 782–787.
3. Krieg, A. M. (2000) The role of CpG motifs in innate immunity. *Curr. Opin. Immunol.* **12**, 35–43.
4. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
5. Hacker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., and Wagner, H. (2000) Immune Cell Activation by Bacterial CpG-DNA through Myeloid Differentiation Marker 88 and Tumor Necrosis Factor Receptor-Associated Factor (TRAF)6. *J. Exp. Med.* **192**, 595–600.
6. Krieg, A. M., Yi, A.-K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B cell activation. *Nature* **374**, 546–549.
7. Wagner, H. (1999) Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol.* **73**, 329–368.
8. Pisetsky, D. S. (1996) Immune activation by bacterial DNA: A new genetic code. *Immunity* **5**, 303–310.
9. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nature Medicine* **3**, 849–854.
10. Lipford, G. B., Sparwasser, T., Bauer, M., et al. (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* **27**, 3420–3426.
11. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3049.
12. Sparwasser, T., Miethke, T., Lipford, G., et al. (1997) Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor- α -mediated shock. *Eur. J. Immunol.* **27**, 1671–1679.
13. Iho, S., Yamamoto, T., Takahashi, T., Yamamoto, S. (1999) Oligodeoxynucleotides containing palindrome sequences with internal 5'- CpG-3' act directly on human NK and activated T cells to induce IFN- γ production in vitro. *J. Immunol.* **163**, 3642–3652.

14. Gilkeson, G. S., Conover, J., Halpern, M., Pisetsky, D. S., Feagin, A., Klinman, D. M. (1998) Effects of bacterial DNA on cytokine production by (NZB/NZW)F₁ mice. *J. Immunol.* **161**, 3890–3895.
15. Halpern, M. D., Kurlander, R. J., and Pisetsky, D. S. (1996) Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell. Immunol.* **167**, 72–78.
16. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* **29**, 2344–2353.
17. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
18. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M. D., Kornbluth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
19. Askew, D., Chu, R. S., Krieg, A. M., and Harding, C. V. (2000) CpG DNA induces maturation of dendritic cells with distinct effects on nascent and recycling MHC-II antigen processing mechanisms. *J. Immunol.* **165**, 6889–6895.
20. Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999) CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* **96**, 9305–9310.
21. Schattenberg, D., Schott, M., Reindl, G., Krueger, T., Tschoepe, D., Feldkamp, J., Scherbaum, W. A., Seissler, J. (2000) Response of Human Monocyte-Derived Dendritic cells to Immunostimulatory DNA. *Eur. J. Immunol.* **30**, 2824–2831.
22. Chu, R., Targoni, O. S., Krieg, A. M., Lehmann, P. V., Harding, C. V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* **186**, 1623–1631.
23. Oxenius, A., Martinic, M. M., Hengartner, H., Klenerman, P. (1999) CpG-containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. *J. Virol.* **73**, 4120–4126.
24. Vabulas, R. M., Pircher, H., Lipford, G. B., Hacker, H., Wagner, H. (2000) CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *Journal of Immunology* **164**, 2372–2378.
25. Chu, R. S., McCool, T., Greenspan, N. S., Schreiber, J. R., and Harding, C. V. (2000) CpG oligodeoxynucleotides act as adjuvants for pneumococcal polysaccharide-protein conjugate vaccines and enhance anti-polysaccharide immunoglobulin G2a (IgG2a) and IgG3 antibodies. *Infect. and Immun.* **68**, 1450–1456.
26. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344.

27. Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J., Krieg, A. M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
28. Moldoveanu, Z., Love-Homan, L., Huang, W. Q., Krieg, A. M. (1998) CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* **16**, 1216–1224.
29. Sun, S., Kishimoto, H., Sprent, J. (1998) DNA as an adjuvant: capacity of insect DNA and synthetic oligodeoxynucleotides to augment T cell responses to specific antigen. *J. Exp. Med.* **187**, 1145–1150.
30. McCluskie, M. J., Davis, H. L. (1998) CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* **161**, 4463–4466.
31. Brazolot Millan, C. L., Weeratna, R., Krieg, A. M., Siegrist, C. A., and Davis, H. L. (1998) CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl. Acad. Sci. USA* **95**, 15,553–15,558.
32. Klinman, D. M., Barnhart, K. M., Conover, J. (1999) CpG motifs as immune adjuvants. *Vaccine* **17**, 19–25.
33. Horner, A. A., Ronaghy, A., Cheng, P. M., et al. (1998) Immunostimulatory DNA is a potent mucosal adjuvant. *Cell Immunol.* **190**, 77–82.
34. Zimmermann, S., Egeter, O., Hausmann, S., et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* **160**, 3627–3630.
35. Walker, P. S., Scharon-Kersten, T., Krieg, A. M., et al. (1999) Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN- γ -dependent mechanisms. *Proc. Natl. Acad. Sci. USA* **96**, 6970–6975.
36. Chiamonte, M. G., Hesse, M., Cheever, A. W., Wynn, T. A. (2000) CpG oligonucleotides can prophylactically immunize against Th2-mediated schistosoma egg-induced pathology by an IL-12-independent mechanism. *J. Immunol.* **164**, 973–985.
37. Broide, D., Schwarze, J., Tighe, H., et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054–7062.
38. Serebrisky, D., Teper, A. A., Huang, C. K., et al. (2000) CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J. Immunol.* **165**, 5906–5912.
39. Chu, R. S., Askew, D., Noss, E. H., Tobian, A., Krieg, A. M., and Harding, C. V. (1999) CpG oligodeoxynucleotides down-regulate macrophage class II MHC antigen processing. *J. Immunol.* **163**, 1188–1194.
40. Cella, M., Engering, A., Pinet, V., Pieters, J., and Lanzavecchia, A. (1997) Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **388**, 782–787.
41. Rescigno, M., Citterio, S., Thery, C., et al. (1998) Bacteria-induced neo-biosynthesis, stabilization, and surface expression of functional class I molecules in mouse dendritic cells. *Proc. Nat. Acad. Sci. (USA)* **95**, 5229–5234.

42. Griffin, J. P., Chu, R., Harding, C. V. (1997) Early endosomes and a late endocytic compartment generate different peptide-class II MHC complexes via distinct processing mechanisms. *J. Immunol.* **158**, 1523–1532.

Activation of B cells by CpG Motifs in Bacterial DNA

Ae-Kyung Yi and Arthur M. Krieg

1. INTRODUCTION

Although the immune system possesses exquisitely antigen-specific receptors on T cells and B cells, the generation of such adaptive immune responses relies on the assistance of the innate immune defenses, such as dendritic cells, which must be activated in order to trigger optimal immune responses. These cells of the innate immune system lack such highly specific antigen receptors, instead relying on a set of “pattern recognition receptors” (PRRs) which have a general ability to detect certain molecular structures that are common to many pathogen occasions, but are not present in self tissues (1,2). One such PRR is represented by unmethylated CpG dinucleotides in particular base contexts, which are prevalent in bacterial and many viral DNAs, but are heavily suppressed and methylated in vertebrate genomes (1–5). Thus, the immune system appears to use the presence of this molecular structure as a “danger signal” that indicates the presence of infection and activates appropriate defense pathways. B cells are one of a very small subset of immune cells that express TLR-9, the putative CpG receptor, giving them a key role in the initiation of CpG-induced immune responses. The purpose of this review is to examine the B-cell effects of CpG DNA, and consider how these may affect the activation of innate and acquired immunity.

2. CELLULAR IMMUNOLOGY OF CpG DNA

2.1. *CpG DNA: a B Cell Mitogen*

Optimal CpG sequences are extraordinarily strong mitogens for murine, bovine, and human B cells. (4,6–9) CpG ODN can drive more than 95% of

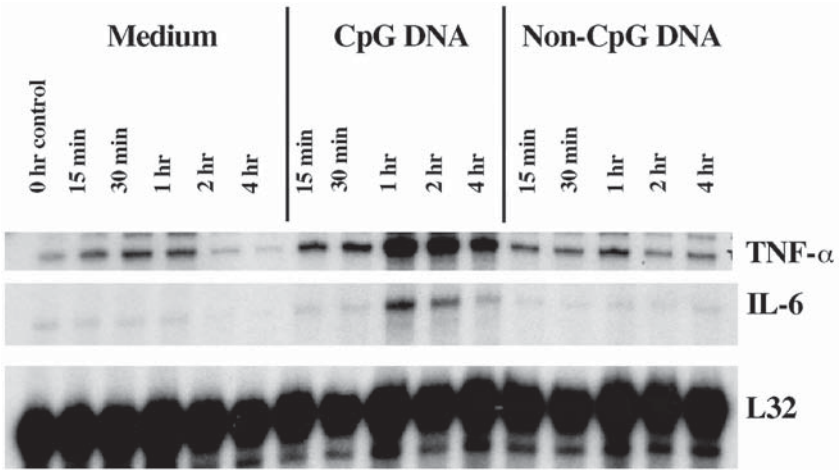


Fig. 1. CpG DNA induces spleen B-cell cytokine gene expression. Spleen B cells isolated from B₆D₂F₁ mice were stimulated with medium, CpG S-ODN (ODN 1668, 1 μ M) or non-CpG S-ODN (ODN1745, 1 μ M) for various periods indicated. Total RNA was isolated and mRNA levels of cytokines were analyzed using multi-probe Rnase Protection Assay as described previously (25). L32 was used for a loading control.

B cells into the cell cycle as compared to only about 80% of all B cells using optimal concentrations of lipopolysaccharide (LPS) (4). Some B cell mitogens, such as 8-substituted guanines, preferentially effect the large, activated subset of B cells (10,11). However, CpG DNA is essentially equally stimulatory to both resting and activated B cells (12). Both the B1 and B2 subsets of B cells are strongly activated by CpG DNA, and enter the G1 phase of the cell cycle within a few hours.

2.2. CpG DNA and B Cell Cytokines

In addition to inducing B cells to proliferate, CpG DNA induces B cells to secrete several cytokines, including tumor necrosis factor (TNF)- α , IL-6, and IL-10, within a few hours (13–15) (Fig. 1) The IL-6 induction is required in order for the B cells to proceed to secrete immunoglobulin-M (IgM) (13). This IL-6 and subsequent IgM secretion in response to CpG DNA is enhanced by another CpG-induced cytokine, IFN- γ . LPS-induced B-cell secretion of IgM is inhibited by addition of interferon (IFN)- γ (16). In contrast, CpG-induced B cell secretion of IL-6 and IgM is more than doubled by the addition of exogenous IFN- γ . This costimulatory interaction also occurs in vivo, since mice genetically deficient in IFN- γ produce less than half of

the IL-6 and IgM response to CpG DNA as occurs in wild-type mice (17). This CpG-induced IFN- γ secretion appears to be derived from natural killer (NK) cells and is dependent on IL-12 production by monocytic cells (18).

The production of IL-10 in response to CpG DNA appears to function as a counter-regulatory mechanism that downregulates the general Th1-like cytokine response. Both in vitro and in vivo, this IL-10 reduces the magnitude and duration of IL-12 secretion (14,19). This counter-regulatory mechanism appears to be responsible for the otherwise paradoxical costimulatory effect of Cyclosporin-A (CsA) on CpG-induced IL-12 production (14). CsA blocks the CpG-induced B-cell secretion of IL-10, but does not block the CpG-induced macrophage production of IL-12 (14). As a result, CsA prevents the “off” signal from IL-10, resulting in an approximate doubling of the IL-12 response to CpG in vitro and in vivo (14). In concert with cytokines, CpG DNA contributes to the human germinal center (GC) B-cell differentiation. CpG DNA enhances plasma cell generation from all the human B-cell subsets in the presence of IL-10, whereas it amplifies the proliferation of naive and memory B cells in the presence of IL-4. CpG DNA, however, does not modulate the generation of memory B cells from GC B cells (Jung J. et al., manuscript submitted).

2.3. Expression of Costimulatory Molecules on CpG-Activated B Cells

In addition to secreting cytokines and Ig, CpG-activated B cells express increased levels of the Fc γ receptor and costimulatory molecules such as class II MHC, CD80, and CD86 (4,20,21). These effects are not unique to rodent B cells, since human B cells upregulate not only these costimulatory molecules, but also CD40 and CD54 (7). CpG DNA also activates malignant B cells from patients with chronic lymphocytic leukemia, driving them into the cell cycle, inducing cytokine secretion, and upregulating surface expression of CD40, CD58, CD80, CD86, CD54, and MHC class I (22).

2.4. Interaction with Signals Activated by Other Receptors in Innate and Adaptive Immunity

Unlike LPS, CpG DNA does not require any serum factor to amplify its B-cell activation. Moreover, C3H/HeJ mice, which are insensitive to LPS, show normal responses to CpG DNA (4). Recent studies using gene deficient mice revealed that LPS and CpG DNA utilize different PRRs, TLR-4 and TLR-9, respectively (23,24). Because upon binding to their ligands both TLR-4 and TLR-9 recruit the same signaling modulators including MyD88, IRAK, and TRAF6, in monocytic cells, CpG DNA and LPS have been suggested to share one or more common signaling pathways for immune cell

activation (23,24). Like innate immune cells such as macrophages/monocytes and DCs, B cells express several different TLRs, including TLR-9, as well as MyD88, IRAK, and TRAF6 (A.K. Yi and SC Hong, unpublished data). However, whether CpG DNA utilizes a MyD88/IRAK/TRAF6 pathway alone or with other signaling pathways in B-cell activation has not been reported. Interestingly, CpG and LPS synergize for induction of B-cell proliferation, Ig secretion (Fig. 2), and cytokine production, providing evidence that these B cell mitogens work through distinct molecular pathways (Yi, A. K. and Krieg, A. M. manuscript in preparation).

The signals induced by CpG DNA can either oppose or enhance signals induced by activation of the B-cell antigen receptor (BCR). In mature peripheral B cells, low concentrations of CpG DNA strongly synergize with signals through the BCR, leading to an approx 10-fold increase in B-cell proliferation and antigen-specific Ig secretion (4). CpG DNA also synergizes with the BCR for induction of cytokine secretion including TNF- α , IL-6, and IL-10 in both mature and immature B cells (13) (and A.K. Yi and A.M. Krieg, manuscript in preparation). This synergy between CpG and the BCR is also evident at the stage of activation of upstream signaling modulators including p38 and c-Jun terminal kinase (JNK) as well as their direct upstream effectors such as MKK3, MKK4, and MKK6. However, CpG and BCR do not synergize for ERK activation (Yi, A. K. and Krieg, A. M. manuscript in preparation).

2.5. Anti-Apoptotic Effects of CpG DNA

In contrast to the synergy between CpG and the BCR in mature B cells, CpG DNA can oppose B cell apoptosis that is triggered by crosslinking of the BCR in certain B-cell lines such as WEHI-231 (25). WEHI-231 B cells have an immature phenotype and are often used as a model for immature B cells that are induced to undergo apoptosis by BCR crosslinking (26) although differences in the signaling pathway used by primary immature B cells and WEHI-231 B cells have been reported (27). In any case, BCR crosslinking in these B cells leads to decreased *c-myc* levels and NF κ B activity unless the B cells are rescued by second signals such as LPS or CD40L. (28–32) CpG DNA also acts as a second signal to protect WEHI-231 B cells against BCR-induced apoptosis through a mechanism that is associated with the maintenance of NF κ B p50/c-Rel heterodimer levels and *c-myc* and *bcl-xL* expression (25,33).

The anti-apoptotic effect of CpG DNA on WEHI-231 cells has recently been extended to another murine B cell lymphoma, BKS-2 (34). These investigators demonstrated a similar association of apoptosis protection with NF κ B induction. In addition, CpG DNA is shown to induce expression of

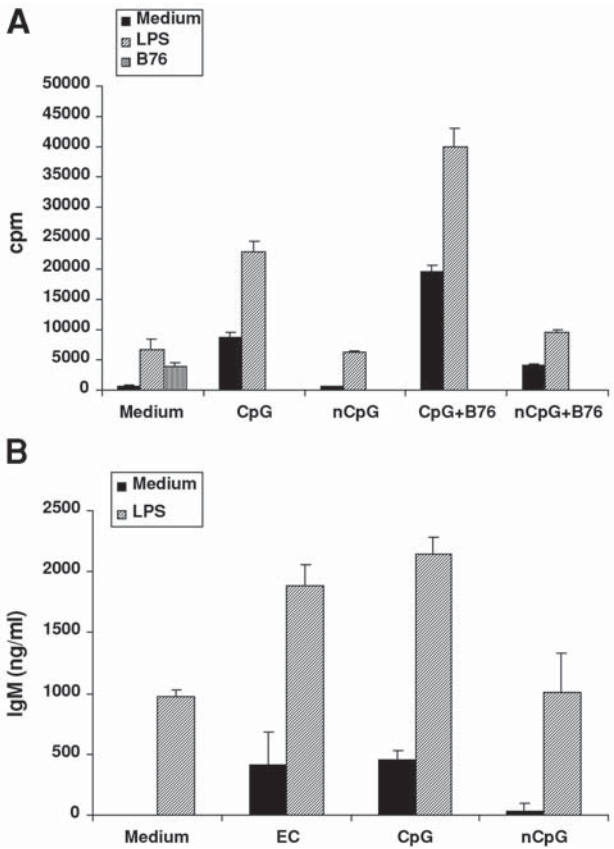


Fig. 2. CpG DNA and LPS synergize for B-cell proliferation and IgM secretion. **(A)** CpG DNA and LPS synergize for spleen B cell proliferation. Spleen B cells isolated from DBA/2 mice (5×10^4 cells/well) were stimulated with medium, B76 (anti-IgM, μ -chain specific monoclonal antibody, 10 μ g/mL), CpG ODN (ODN 684, 20 μ M), non-CpG ODN (nCpG, ODN1471, 20 μ M), or combination of B76 and ODN in the presence or absence of LPS (1 μ g/mL) for 44 h with last 4 h pulse with 2 μ Ci/well of [^{3} H]-Uridine. **(B)** CpG DNA and LPS synergized for spleen B-cell IgM production. Spleen B cells isolated from DBA/2 mice (10^5 cells/well) were stimulated with medium, *E. coli* DNA (EC, 50 μ g/mL), CpG ODN (ODN 684, 20 μ M), or non-CpG ODN (nCpG, ODN1471, 20 μ M) in the presence or absence of LPS (1 μ g/mL) for six days. Levels of IgM in the culture supernatants were measured by IgM-specific ELISA.

egr-1 mRNA in BKS-2 cells, whereas an antisense oligodeoxynucleotide (ODN) against *egr-1* blocks the CpG-induced protection against apoptosis (34). Although this experiment was interpreted to suggest a role for *egr-1* in

mediating the protective effect of CpG DNA, there is a potential complication. The antisense sequence used for *egr-1* contained several poly-G motifs. Since poly-G motifs can oppose the immune stimulatory effects of CpG ODN (35), and since these motifs can also have direct antiproliferative activities on cell lines (36,37), it remains possible that the observed effect of these ODNs may not be due to an antisense mechanism of action.

Mature primary B cells undergo spontaneous apoptosis in tissue culture unless they are rescued by growth factors or other mitogens (38,39). CpG DNA also prevents this spontaneous apoptosis by maintaining NFkB activation (40). This protective effect is associated with increased levels of *c-myc*, *egr-1*, *c-Jun*, *bcl_{XL}*, and *bax* mRNA. Inhibition of protein synthesis only partially reduced the apoptosis protection. CpG DNA prevents the normal reduction of the mitochondrial membrane potential that is associated with spontaneous apoptosis of primary B cells (41) CpG DNA has also been reported to protect B cells against Fas-mediated apoptosis by downregulating Fas expression on B cells stimulated through CD40 (42).

3. MECHANISMS OF ACTION

3.1. DNA Backbone

The strongest effects of CpG DNA on B cells are seen with the use of ODN synthesized with a nuclease resistant phosphorothioate backbone (PS). ODN constructed with the normal phosphodiester DNA backbone (PO) are rapidly degraded inside lymphocytes (43). Replacement of one of the nonbridging oxygen atoms around the PO linkage with a sulfur atom, which is called a PS linkage, leads to an extremely high degree of nuclease resistance, which greatly stabilizes the ODN against degradation and dramatically enhances the immunostimulatory activity of the ODN if it contains a CpG dinucleotide (43–46). In fact, PS CpG ODN are approx 200 times more potent for activating murine B cell proliferation than the corresponding PO CpG ODN (4,40). Human B cells also are activated to a much greater degree by PS CpG ODN compared to PO (6). The phosphorothioate backbone modification creates a chiral center at each internucleotide linkage. In vivo, the S-isomer is highly resistant to nuclease degradation, but the R-isomer is degraded at a more rapid rate than a stereo-random ODN. Consistent with their difference in stability, CpG ODN synthesized with an R backbone are less potent at inducing B-cell proliferation compared to the S isomer (47).

The susceptibility of PO ODN to degradation not only reduces their ability to drive B-cell proliferation, but can even result in an artifact in studies using a ³H thymidine incorporation assay to measure B cell proliferation. In

such assays, phosphodiester DNA is degraded, releasing free thymidine which competes for the ^3H thymidine and causes a false suppression of incorporation (48). This nonspecific effect is most marked if thymidine nucleotides are present at the 3' end of an ODN, but can also be observed with high molecular weight DNA such as bacterial genomic DNA. Certain exonuclease activities are specific to B lymphocytes, indicating that results obtained using PO DNA in one cell type cannot necessarily be extrapolated to other cell types (49). Moreover, nuclease activities appear to be higher in human than in mouse cells, with the result that phosphodiester DNA can appear to be nonstimulatory unless it is added repeatedly (50).

The PS ODN backbone changes the biological properties of the ODN compared to normal PO DNA. The nonspecific binding to a wide variety of proteins is dramatically increased (51,52). Phosphorothioate ODN bind much more avidly to cell membranes, and generally have a much higher degree of cell uptake compared to PO (43,53,54). The PS backbone may have certain sequence independent immune stimulatory activities such as the activation of SP1 transcription factor activity (55), inhibition of smooth muscle cell proliferation and migration (56,37), inhibition of basic fibroblast growth factor binding to its receptor (57,58) and angiogenic activity (59), reduction of the sequence specific binding of transcription factors to their binding sites (60), inhibition of cellular adhesion to extracellular matrix (61), enhancement of LPS-induced TNF production (62), and some degree of non-sequence specific immune stimulation (63). The immune stimulatory effects are reduced by further modification with 2' methoxyethoxy (64). Finally, the PS backbone enhances certain effects of poly G sequences, which may form G quartets, including the ability to inhibit CD28 expression and in vivo contact hypersensitivity responses (65).

There are some differences in the immune stimulatory effects of CpG motifs in PS ODN compared to PO ODN. Although the optimal CpG motif for driving murine B-cell proliferation has the formula, purine-purine-CG-pyrimidine-pyrimidine, PO ODN bearing sub-optimal CpG motifs, such as those in which the CG is preceded by a C or followed by a G, can still drive B-cell proliferation if the ODN concentration is increased (4,40). In contrast, apart from the nonspecific effects of the thioate backbone, PS ODN bearing suboptimal CpG motifs are less likely to drive high levels of B-cell proliferation, especially if the CG is followed by a G. PS ODN without CG motifs are also frequently observed to drive the proliferation of murine and human B cells, although to a more limited degree than that which occurs with CpG ODN (40,6). However, this broad B cell stimulatory activity of the PS ODN backbone shows some sequence dependence, as PS ODN with

certain types of CpG motifs actually exert a neutralizing activity that diminishes the proliferative response to a stimulatory CpG motif (66–68). PS ODN comprised solely of CG dinucleotides or those in which the CGs are preceded by a C and/or followed by a G appear to be the most potent neutralizing motifs. As a rule, PS ODN are much more potent at activating B cells compared to the same sequence with a PO backbone (4). In contrast, PS ODN are generally less active at activating macrophages or NK cells, compared to ODN in which at least part of the backbone is PO (35,69).

Besides the CpG motif, another type of sequence motif independently modulates the PS backbone and CpG motif effect on B cells. This motif has been termed a poly G motif, or G tetrad, and consists of one or more runs of three or preferably four consecutive Gs (37). Poly G motifs in ODN form complex higher ordered structures (“G tetrads”) that on the one hand enhance ODN uptake by cells through binding to the scavenger receptor (in the case of macrophages) and/or direct binding to the lipid bilayer of the cell membrane (70). On the other hand, poly G sequences have independent immune activities, including enhancing B-cell proliferation, but not cytokine production (71). Based on the differential effects of the PS backbone and poly G sequences on CpG-induced B-cell activation, we have defined two distinct classes of immune stimulatory ODN (Table 1). CpG-A ODN are optimal for activating NK cells and inducing IFN- γ expression from plasmacytoid dendritic cells, but have relatively little mitogenic activity for B cells and do not stimulate B cell cytokine production (72,35). The failure of CpG-A ODN to strongly activate B-cells is attributable to the use of a PO backbone for the CpG part of the ODN, and to the incorporation of inhibitory Poly G motifs, that block B-cell activation. The most potent B-cell stimulation is seen with CpG-B type ODN, that have a completely PS backbone, without inhibitory sequences.

3.2. Cellular Binding

The ability of cell membranes to bind DNA has been recognized for many years (73,74). Bennett and colleagues and Jacob et al. have reported the existence of several cell surface DNA binding proteins which bind high molecular weight DNA and appear to be autoantigens in lupus (75–79). The identity of most of these surface DNA-binding proteins has remained unclear. Two groups have reported a 79–80 kD ODN-binding protein in cell membranes, with one group also detecting a 90 kD band (80,81). The leukocyte integrin Mac1 has been reported to be an ODN-binding protein, but this would not be relevant to B cells, which do not express it (82). Others have reported that B cells take up DNA through their surface immunoglobulin,

Table 1
Distinct Immune Effects of Different CpG ODN

Class	Backbone ^a	Poly G motifs ^b	Palindrome ^c	B cell ^d	NK cell ^e	DC ^f	Macrophage IL-12 promoter ^g	Macrophage HIV LRT promoter ^h
CpG-A	O, SOS	Enhance stimulation ^(35,70)	Enhance ⁽¹²⁶⁾	+ ^(4,46)	++++ ^(35,126,127)	++++ ^(50,121)	++ ⁽¹²⁸⁾	+++ ⁽¹²⁸⁾
CpG-B	S	Suppress stimulation ⁽³⁵⁾	Not required ⁽⁴⁾	++++ ^(4,46)	+ ^(35,127)	++++ ^(50,121)	++ ++ ⁽¹²⁸⁾	+/- ⁽¹²⁸⁾

^aBackbones of the DNA include native phosphodiester (O), nuclease-resistant phosphorothioate (S), or chimeric, where only the linkages at the 5' and 3' ends are S⁽³⁵⁾ addition to greatly increasing the stability of the DNA, the S backbone enhances cell uptake compared to O DNA.

^bPoly G motifs typically contain 4 or more consecutive Gs, and can form higher ordered structures called G tetrads, that have independent immune activities^(37,129-131) may enhance cell uptake of the ODN⁽⁷⁰⁾, and/or interact with other cellular proteins.

^crefers to the presence of a sequence that is self complementary such as AACGTT (potentially capable of formation of duplex DNA structures)

^dproliferation, expression of CD80, CD86, Ig and IL-6 secretion

^eIFN- γ secretion, ability to lyse target cells

^fexpression of class II MHC, CD80, CD86

^gCpG-containing S-ODN are active at 10- to 100-fold lower concentrations than corresponding O-ODN in maintenance of macrophage viability in the absence of CSF-1, in induction of NO production, and in activation of the IL-12 promoter

^hO-ODN are far more active than corresponding S-ODN at inducing down-modulation of the CSF-1R from primary macrophages, inducing rapid phosphorylation of the extracellular signal-related kinases 1 and 2, and at activating transcription driven by the HIV-1 LTR.

but this has not been confirmed (83). Other investigators have also reported the existence of lymphocyte cell surface binding of nuclear antigens, which is inducible with IL-1 β or LPS, but not by IFN- γ (84). It is unclear whether this is related to DNA binding. Neither we nor other investigators have detected any sequence specificity in the cell surface binding of stimulatory CpG and nonstimulatory ODN (4,85).

The existence of cell surface DNA binding proteins suggests the possibility that the immune stimulatory effects of CpG DNA might be mediated through binding to a membrane receptor. Our initial studies in murine cells indicated that CpG ODN immobilized on a solid support could not activate lymphocytes, which indicated that cell uptake was required (4). In contrast, other investigators have reported that human B cells are stimulated by CpG ODN immobilized on sepharose beads (6). Although these latter experiments appeared to suggest that CpG ODN may work through a cell surface receptor, Manzel and MacFarlane have recently found that ODN coupled to sepharose beads can still be taken up by cells in tissue culture, leaving open the possibility that CpG ODN may still work through an intracellular signaling pathway rather than a cell surface receptor (86). These latter investigators went on to show that CpG ODN which were linked to latex, magnetic, or gold beads could not be taken up and lost their stimulatory activity. Moreover, lipofection of ODN into spleen cells enhances their immune stimulatory effects and makes it possible for very short ODN, which normally are nonstimulatory, to exert a CpG sequence-dependent stimulatory effect (85).

3.3. Cellular Uptake

None of these studies reviewed previously have demonstrated any role for the cell surface DNA binding proteins in mediating the cellular uptake of DNA. However, Klotman and colleagues identified a 45 kD protein present in kidney brush border membranes which appears to function as a voltage-gated channel for the entry of ODN into these cells (87). There is no evidence that this protein is expressed in lymphocytes, so it may not be related to the immune stimulatory effects of CpG DNA, although it points to the possibility that ODN may have effects on other tissues.

In B cells, the mechanism of ODN uptake remains far from certain. It is clear that ODN uptake is an active process which is temperature and energy dependent, competable, saturable, and is generally sequence-independent with the exception that G-rich sequences can enhance the uptake of PO ODN (54,70,88,89). Given that ODN cannot diffuse across cell membranes, possible mechanisms for their uptake include phagocytosis, pinocytosis, potocytosis, and several different types of endocytic pathways including

adsorptive endocytosis and receptor-mediated endocytosis, which can be mediated through clathrin-coated pits or may be clathrin-independent. The fact that many nonphagocytic cells readily internalize ODN demonstrates that this uptake process cannot be essential. Pinocytosis has been reported to be important in cell uptake at relatively high ODN concentrations ($>1\ \mu\text{M}$) whereas receptor-mediated endocytosis, possibly involving clathrin, may be most important at lower ODN concentrations (81,90). Measures to enhance the endosomal release of ODN, such as delivery with cationic liposomes, conjugation to cholesterol, or the use of endosome-disrupting agents enhance the immune stimulatory effect of CpG ODN (91,85).

The rates of ODN uptake differ dramatically between different cell subpopulations. For example, T cells generally have a low rate of ODN uptake compared to B cells or monocytic cells in *in vitro* studies (92–95). However, uptake also appears to be regulated depending on the stage of cell differentiation, at least in B cells (53). In lymphocytes, ODN uptake is highly inducible by mitogens (92,93). ODN uptake in malignant cells and cell lines is typically higher than that in primary cells, which may provide an extra therapeutic margin for the development of ODN-based therapies of malignant disease (93).

The *in vivo* uptake of ODN has been studied using a variety of approaches. Studies using ^{35}S -labeled PS ODN have been useful in delineating the organ distribution of administered ODN, though not differences in uptake between organs. These studies demonstrate that the main organs of distribution for S-ODN are the liver and kidney, with lower levels in the spleen and bone marrow (reviewed in 96). Biodistribution is similar after IV or SC administration, but oral absorption is poor, with approx 0.1% of the ingested dose becoming systemically absorbed. The cellular distribution of ODN after IV administration was examined by Zhao et al., using FITC-conjugated ODN (97). These experiments demonstrated that ODN uptake was quite heterogeneous among peripheral blood mononuclear cells (PBMC), spleen, and bone marrow cells, with highest levels of uptake in monocytes and macrophages, intermediate levels in B cells, and low levels in T cells.

3.4. Requirement for Endosomal Acidification/Maturation

One of the unanswered questions in the intracellular trafficking of ODNs is how they are apparently able to exit the endosomes. It is possible that this has something to do with endosomal acidification or other aspects of the intracellular endosomal maturation. To address this possibility, we tested whether monensin, chloroquine, and bafilomycin A, which interfere with

endosomal acidification and/or maturation, would affect CpG-induced activation. Surprisingly, these compounds completely blocked the ability of CpG DNA to activate B cells or monocytic cells at low concentrations which did not inhibit stimulation by LPS, anti-CD40, crosslinking of the B-cell antigen receptor, or by phorbol 12-myristate 13-acetate (98). Other compounds structurally related to chloroquine, such as quinacrine, are even more potent antagonists of CpG-induced immune stimulation (99,100). These compounds appear to act at an extremely early step in the CpG-induced signaling pathway, since their inhibitory effects are already apparent by five minutes, and since they block all of the signaling pathways yet known to be induced by CpG (98,101,102).

3.5. Requirement for TLR-9 in B-cell Responses to CpG DNA

Recently, the immune activation by CpG DNA has been shown to depend on MyD88, which is known as an adapter protein for the toll-like receptor (TLR) family (103,104). This suggested the likelihood that a member of the TLR family may mediate immune activation by CpG DNA, and this has been confirmed with the demonstration of a requirement for TLR-9 in B cell and DC responses to a PS CpG ODN (23). Although it seems unlikely, it remains possible that activation by bacterial DNA or PO CpG motifs may differ. Among human immune cell types, expression of TLR-9 has only been clearly demonstrated in B cells and plasmacytoid DC, and therefore correlates with responsiveness to direct stimulation by CpG DNA (105). TLR-9 has a transmembrane domain, and other features of a type I membrane protein (106,107). However, chloroquine and related endosomal disrupting agents block CpG signaling (41,98,99,101,102), providing further evidence that some further steps must be required for CpG signaling, beyond cell membrane binding. TLR-9 may interact with CpG DNA in the ER, instead of at the cell surface (105). The species specificity of CpG motif recognition appears to be determined by TLR-9, providing indirect evidence that it may make direct contacts with the CpG motif (105). There is as yet no evidence that TLR-9 is required for DNA uptake, though the existence of multiple potential mechanisms for ODN uptake makes it difficult to exclude a contributory role for TLR-9 (*see Subheading 3.6.*).

3.6. Activation of the Mitogen Activated Protein Kinase Pathways

Mitogen-activated protein kinases (MAPKs) are common pathways which have an important role in leukocyte responses to diverse stimuli. Three principal MAPK pathways can be distinguished, including the extracellular receptor kinase pathway (ERK), the p38 MAPK pathway, and the c-Jun NH₂-terminal kinase (JNK) pathway. B cells activated by CpG

DNA show activation of both the p38 and JNK pathways but not ERK within seven minutes (102). In comparison to B cell activation by CD40 ligation, CpG DNA showed a slightly slower onset of MAPK phosphorylation, but a longer duration of activation. Interestingly, the JNK isoforms phosphorylated in response to CpG DNA had apparently identical molecular weights to those phosphorylated in response to CD40 ligation, but were different from those seen in B cells activated through the BCR (102). Human B cells activated by CpG DNA also show activation of the p38 and JNK MAPKs, but not ERK (7). The p38 pathway appears to be required for CpG-induced B cell cytokine secretion, since this is completely blocked by pretreatment of the cells with a p38 inhibitor (102). However, p38 does not appear to be required for the CpG DNA-mediated B-cell proliferation or protection against either BCR-induced apoptosis of immature B cells or spontaneous apoptosis of mature splenic B cells (Yi, A. K. and Krieg, A. M., manuscript in preparation).

The p38 and JNK pathways also are rapidly activated in macrophages following exposure to CpG DNA (101,102). CpG-induced p38 activity promotes production of TNF- α , IL-6, and IL-12p40 in macrophage and DCs. Of note, CpG also activates the ERK pathway in primary macrophages and a macrophage-like cell line, RAW264.7, which contributes to CpG-induced TNF- α production (108) (Yi, Yoon, et al. manuscript in preparation). However, CpG DNA does not induce activation of ERK in primary DCs or a different macrophage-like cell line, J774 (102,108). In macrophages, this CpG-induced ERK activity has a negative feedback effect on the IL-12 p40 promoter, resulting in decreased release of IL-12. In order to test the hypothesis that ERK activation may have a negative feedback effect on CpG-induced IL-12 synthesis, Hacker et al. chemically activated the ERK pathway in CpG-induced DCs. This resulted in suppressed production of IL-12 (108). Studies using IL-10 gene deficient mice indicates that this ERK-mediated suppression of the CpG DNA-induced IL-12 production is owing to production of IL-10 that is promoted by the activated ERK (108a). These studies suggest that the activation of p38 by CpG DNA contributes to IL-12 production, but that ERK activation can exert a negative feedback effect. The biologic role of CpG-induced JNK activation in cytokine production by monocytic cells as well as B cells is currently unknown.

3.7. Activation of Nuclear Factor- κ B

Nuclear factor- κ B (NF- κ B) is rapidly upregulated in leukocytes that have been exposed to a broad range of stimuli, and is thought to have a critical role in regulating inflammatory responses (109,110). Given the impressive proinflammatory effects of CpG DNA, it may be expected that it should

trigger NF- κ B activation. Indeed, even prior to the discovery of the CpG motif, McIntyre et al. reported the unexpected observation that in an intended antisense experiment, a control sense ODN to the p65 subunit of NF- κ B caused a dramatic upregulation of NF- κ B activity in murine splenic B cells (111). This control sense ODN to p65 contained an immune stimulatory CpG motif, which was responsible for the observed upregulation of NF- κ B activity (A.M. Krieg, R. Narayanan, unpublished data). Further studies have demonstrated that CpG DNA activates NF- κ B in both macrophages and B cells (112,33). This NF- κ B activation was associated with the degradation of I κ B α and I κ B β (33). In B cells, the dominant form of NF- κ B induced by CpG DNA appears to be a p50/c-Rel heterodimer, although in macrophages it appears to be a p50/p65 heterodimer (33,113). This NF- κ B activation results in enhanced transcriptional activity from the human immunodeficiency virus long-terminal repeat (112), and is required for the CpG-induced cytokine production in B cells (98) for the protection of B cells against apoptosis induced by BCR ligation (33), and for the protection of primary B cells from spontaneous apoptosis in tissue culture (41). CpG-induced NF- κ B activation is also seen in human primary B cells (7).

The mechanism through which CpG DNA induces NF- κ B in B cells is unclear. One possibility is related to the production of reactive oxygen species (ROS) which is increased within five minutes of CpG DNA treatment (13). NF- κ B activation is known to be highly dependent on the redox state of the cell (114,115) suggesting a possible link to the CpG-induced oxidative burst. CpG-induced NF- κ B activation is blocked by antioxidants, consistent with this hypothesis (98,33).

3.8. Activation of Transcription and Translation

The MAPK pathways which are activated by CpG DNA have been reported in other systems to lead to the activation of multiple transcription factors, including ATF-1, ATF-2, the cyclic AMP response element binding protein (CREB), Elk-1, Max, and c-Jun (116–118). Some of these factors have already been shown to become phosphorylated and activated in response to CpG DNA treatment of B cells and/or macrophages (101,102). Like NF- κ B, which is also activated by CpG DNA and B-cell types, these transcription factors are important regulators for the expression of many cellular protooncogenes and pro-inflammatory cytokines. CpG DNA also induces increased mRNA levels of several other transcription factors, including *c-jun*, *c-fos*, *c-myc*, Ets-2, C/EBP- β and - δ (40,98,119). Cooperation between transcription factors of different classes is thought to be an

important mechanism in the coordinate regulation of gene expression in response to different stimuli.

So far there have been no reported detailed analyses of promoter or enhancer function in CpG-stimulated B cells to identify the binding sites responsible for these transcriptional responses. However, promoter activity for TNF- α , IL-6, and HIV has been shown to be induced by CpG DNA in macrophages and B cells (13,119) (108a). This transcriptional induction appears to be extremely rapid, with increased levels of mRNA within 15 min (40). Among the genes whose RNA expression is increased in B cells are c-myc, c-myn, egr-1, c-jun, bcl-2, bcl-x_L, bax, TNF- α , IL-6, and IL-10 (13,14,25,33,40). Interestingly, although the overall effect of CpG DNA is the strong promotion of Th1-like immune responses, the B-cell production of IL-10 acts to reduce the level of IL-12 secretion that is induced by CpG DNA (14).

CpG DNA also has potent transcription-activating effects on macrophages, leading to the increased transcription of TNF- α , IL-1 β , plasminogen activator inhibitor-2, IL-6, IL-12, Type 1 interferons, and several costimulatory and antigen presenting molecules such as class II MHC, CD80, CD86, and CD40. (19,112,113,120–122). As reviewed previously, NK cells are induced by CpG DNA to produce IFN- γ . Other cytokines whose expression is induced by CpG DNA, but for whom the cellular sources have not yet been conclusively determined, include IL-1RA, MIP-1 β , MCP-1, and IL-18 (123–125).

4. SUMMARY

CpG DNA is a very powerful B-cell mitogen. However, it should be understood that there are tremendous qualitative and quantitative differences in the activity of different CpG DNA sequences; the magnitude of their effects depends on the DNA backbone and the bases flanking the CpG dinucleotide. The ability of the B cell to detect a PRR such as the CpG motif demonstrates clearly the important role of the B cell as a component of innate immune defenses as well as in adaptive immunity. On the other hand, the synergy of this activation pathway with the BCR speeds the development of antigen-specific immune responses and thus enhances the ability of the B cell to protect the host against invasion. With the identification of the role of TLR-9 in mediating B-cell activation by CpG DNA, the field is poised to develop a clearer understanding of the mechanisms leading to the activation of multiple intracellular signaling pathways in CpG-activated B cells.

REFERENCES

1. Dempsey, P. W., Allison, M. E., Akkaraju, S., Goodnow, C. C., and Fearon, D. T. (1996) C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science*. **271**, 348–350.
2. Kumar, A., Yang, Y. L., Flati, V., et al. (1997) Deficient cytokine signaling in mouse embryo fibroblasts with a targeted deletion in the PKR gene: role of IRF-1 and NF-kappaB. *EMBO J.* **16**, 406–416.
3. Bird, A. P. (1987) CpG islands as gene markers in the vertebrate nucleus. *Trends in Genetics*. **3**, 342–347.
4. Krieg, A. M., Yi, A. K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
5. Medzhitov, R. and Janeway, C. A., Jr. (1997) Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* **9**, 4–9.
6. Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S., and Lipsky, P. E. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
7. Hartmann, G. and Krieg, A. M. (2000) Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J. Immunol.* **164**, 944–953.
8. Hartmann, G., Weeratna, R. D., Ballas, Z. K. (2000) Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses *in vitro* and *in vivo*. *J. Immunol.* **164**, 1617–1624.
9. Brown, W. C., Estes, D. M., Chantler, S. E., Kegerreis, K. A., and Suarez, C. E. (1998) DNA and a CpG oligonucleotide derived from *Babesia bovis* are mitogenic for bovine B cells. *Infect. Immun.* **66**, 5423–5432.
10. Wicker, L. S., Boltz, R. C., Jr., Nichols, E. A., Miller, B. J., Sigal, N. H., and Peterson, L. B. (1987) Large, activated B cells are the primary B-cell target of 8-bromoguanosine and 8-mercaptoguanosine. *Cell Immunol.* **106**, 318–329.
11. Goodman, M. G. (1991) Cellular and biochemical studies of substituted guanine ribonucleoside immunostimulants. *Immunopharmacology* **21**, 51–68.
12. Krieg, A. M. (1999) Leukocyte Stimulation by Oligodeoxynucleotides. In: *Applied Antisense Oligonucleotide Technology*, C. A. Stein and A.M. Krieg (eds.), Wiley & Sons, Inc., New York, pp. 431–448.
13. Yi, A. K., Klinman, D. M., Martin, T. L., Matson, S., and Krieg, A. M. (1996) Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* **157**, 5394–5402.
14. Redford, T. W., Yi, A. K., Ward, C. T., and Krieg, A. M. (1998) Cyclosporin A enhances IL-12 production by CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides. *J. Immunol.* **161**, 3930–3935.
15. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis *in vitro*. *Eur. J. Immunol.* **29**, 2344–2353.

16. Chace, J. H., Abed, N. S., Adel, G. L., and Cowdery, J. S. (1993) Regulation of differentiation in CD5+ and conventional B cells. Sensitivity to LPS-induced differentiation and interferon- γ - mediated inhibition of differentiation. *Clin. Immunol. Immunopathol.* **68**, 327–332.
17. Yi, A. K., Chace, J. H., Cowdery, J. S., and Krieg, A. M. (1996) IFN- γ promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J. Immunol.* **156**, 558–564.
18. Cowdery, J. S., Chace, J. H., Yi, A. K., and Krieg, A. M. (1996) Bacterial DNA induces NK cells to produce IFN- γ in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol.* **156**, 4570–4575.
19. Anitescu, M., Chace, J. H., Tuetken, R., et al. (1997) Interleukin-10 functions in vitro and in vivo to inhibit bacterial DNA- induced secretion of interleukin-12. *J. Interferon Cytokine Res.* **17**, 781–788.
20. Davis, H. L., Weeratna, R., Waldschmidt, T. J., et al. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
21. Kobayashi, H., Horner, A. A., Takabayashi, K., Nguyen, M. D., Huang, E., Cinman, N., and Raz, E. (1999) Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1- biased immunity. *Cell Immunol.* **198**, 69–75.
22. Jahrsdorfer, B., Hartmann, G., Racila, E., et al. (2001) CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. *J. Leukoc. Biol.* **69**, 81–88.
23. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature.* **408**, 740–745.
24. Krieg, A. M. and Wagner, H. (2000) Causing a commotion in the blood: immunotherapy progresses from bacteria to bacterial DNA. *Immunol. Today* **21**, 521–526.
25. Yi, A. K., Hornbeck, P., Lafrenz, D. E., and Krieg, A. M. (1996) CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. *J. Immunol.* **157**, 4918–4925.
26. Norvell, A., Mandik, L., and Monroe, J. G. (1995) Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J. Immunol.* **154**, 4404–4413.
27. King, L. B., Norvell, A., and Monroe, J. G. (1999) Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells. *J. Immunol.* **162**, 2655–2662.
28. Lee, H., Arsura, M., Wu, M., Duyao, M., Buckler, A. J., and Sonenshein, G. E. (1995) Role of Rel-related factors in control of c-myc gene transcription in receptor-mediated apoptosis of the murine B cell WEHI 231 line. *J. Exp. Med.* **181**, 1169–1177.
29. Fischer, G., Kent, S. C., Joseph, L., Green, D. R., and Scott, D. W. (1994) Lymphoma models for B cell activation and tolerance. X. Anti- μ - mediated growth arrest and apoptosis of murine B cell lymphomas is prevented by the stabilization of myc. *J. Exp. Med.* **179**, 221–228.

30. McCormack, J.-E., Pepe, V. H., Kent, R. B., Dean, M., Marshak-Rothstein, A., and Sonnenshein, G. E. (1984) Specific regulation of c-myc oncogene expression in a murine B-cell lymphoma. *Proc. Natl. Acad. Sci. USA* **81**, 5546.
31. Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Arsur, M., Katz, D., FitzGerald, M. J., Rothstein, T. L., Sherr, D. H., and Sonenshein, G. E. (1996) Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. *EMBO J.* **15**, 4682–4690.
32. Schauer, S. L., Wang, Z., Sonenshein, G. E., and Rothstein, T. L. (1996) Maintenance of nuclear factor-k B/Rel and c-myc expression during CD40 ligand rescue of WEHI 231 early B cells from receptor-mediated apoptosis through modulation of IkB proteins. *J. Immunol.* **157**, 81–86.
33. Yi, A. K. and Krieg, A. M. (1998) CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of IkBa and IkB β and sustained activation of nuclear factor- κ B/c-Rel. *J. Immunol.* **160**, 1240–1245.
34. Han, S. S., Chung, S. T., Robertson, D. A., Chelvarajan, R. L., and Bondada, S. (1999) CpG oligodeoxynucleotides rescue BKS-2 immature B cell lymphoma from anti-IgM-mediated growth inhibition by up-regulation of egr-1. *Int. Immunol.* **11**, 871–879.
35. Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
36. Yaswen, P., Stampfer, M. R., Ghosh, K., and Cohen, J. S. (1993) Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. *Antisense Res. Dev.* **3**, 67–77.
37. Burgess, T. L., Fisher, E. F., Ross, S. L., et al. (1995) The antiproliferative activity of c-myc and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism. *Proc. Natl. Acad. Sci. USA* **92**, 4051–4055.
38. Illera, V. A., Perandones, C. E., Stunz, L. L., Mower, D. A., Jr., and Ashman, R. F. (1993) Apoptosis in splenic B lymphocytes. Regulation by protein kinase C and IL-4. *J. Immunol.* **151**, 2965–2973.
39. Mower, D. A., Jr., Peckham, D. W., Illera, V. A., Fishbaugh, J. K., Stunz, L. L., and Ashman, R. F. (1994) Decreased membrane phospholipid packing and decreased cell size precede DNA cleavage in mature mouse B cell apoptosis. *J. Immunol.* **152**, 4832–4842.
40. Yi, A. K., Chang, M., Peckham, D. W., Krieg, A. M., and Ashman, R. F. (1998) CpG oligodeoxyribonucleotides rescue mature spleen B cells from spontaneous apoptosis and promote cell cycle entry. *J. Immunol.* **160**, 5898–5906.
41. Yi, A. K., Peckham, D. W., Ashman, R. F., and Krieg, A. M. (1999) CpG DNA rescues B cells from apoptosis by activating NFkappaB and preventing mitochondrial membrane potential disruption via a chloroquine-sensitive pathway. *Int. Immunol.* **11**, 2015–2024.
42. Wang, Z., Karras, J. G., Colarusso, T. P., Foote, L. C., and Rothstein, T. L. (1997) Unmethylated CpG motifs protect murine B lymphocytes against Fas-mediated apoptosis. *Cell Immunol.* **180**, 162–167.

43. Zhao, Q., Matson, S., Herrera, C. J., Fisher, E., Yu, H., and Krieg, A. M. (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res. Dev.* **3**, 53–66.
44. Stein, C. A., Subasinghe, C., Shinozuka, K., and Cohen, J. S. (1988) Physico-chemical properties of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.* **16**, 3209–3221.
45. Zhao, Q., Temsamani, J., Iadarola, P. L., Jiang, Z., and Agrawal, S. (1996) Effect of different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem. Pharmacol.* **51**, 173–182.
46. Krieg, A. M., Matson, S., and Fisher, E. (1996) Oligodeoxynucleotide modifications determine the magnitude of B cell stimulation by CpG motifs. *Antisense Nucleic Acid Drug Dev.* **6**, 133–139.
47. Yu, D., Kandimalla, E. R., Roskey, A., Zhao, Q., Chen, L., Chen, J., and Agrawal, S. (2000) Stereo-enriched phosphorothioate oligodeoxynucleotides: synthesis, biophysical and biological properties [In Process Citation]. *Bioorg. Med. Chem.* **8**, 275–284.
48. Matson, S. and Krieg, A. M. (1992) Nonspecific suppression of [3H]thymidine incorporation by “control” oligonucleotides. *Antisense Res. Dev.* **2**, 325–330.
49. Kenter, A. L. and Tredup, J. (1991) High expression of a 3'–5' exonuclease activity is specific to B lymphocytes. *Mol. Cell Biol.* **11**, 4398–4404.
50. Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999) CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* **96**, 9305–9310.
51. Stein, C. A. and Cheng, Y. C. (1993) Antisense oligonucleotides as therapeutic agents—is the bullet really magical? *Science*. **261**, 1004–1012.
52. Stein, C. A. and Krieg, A. M. (1994) Problems in interpretation of data derived from in vitro and in vivo use of antisense oligodeoxynucleotides [editorial]. *Antisense Res. Dev.* **4**, 67–69.
53. Zhao, Q., Waldschmidt, T., Fisher, E., Herrera, C. J., and Krieg, A. M. (1994) Stage-specific oligonucleotide uptake in murine bone marrow B-cell precursors. *Blood*. **84**, 3660–3666.
54. Crooke, R. M. (1998) In vitro cellular uptake, distribution, and metabolism of oligonucleotides. In: *Antisense Research and Application*, S. T. Crooke (ed.) Springer-Verlag, Heidelberg, Germany, pp. 103–140.
55. Perez, J. R., Li, Y., Stein, C. A., Majumder, S., van Oorschot, A., and Narayanan, R. (1994) Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **91**, 5957–5961.
56. Wang, X. Z. and Ron, D. (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* **272**, 1347–1349.
57. Fennewald, S. M. and Rando, R. F. (1995) Inhibition of high affinity basic fibroblast growth factor binding by oligonucleotides. *J. Biol. Chem.* **270**, 21,718–21,721.

58. Guvakova, M. A., Yakubov, L. A., Vlodavsky, I., Tonkinson, J. L., and Stein, C. A. (1995) Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J. Biol. Chem.* **270**, 2620–2627.
59. Kitajima, I., Unoki, K., and Maruyama, I. (1999) Phosphorothioate oligodeoxynucleotides inhibit basic fibroblast growth factor-induced angiogenesis in vitro and in vivo. *Antisense Nucleic Acid Drug Dev.* **9**, 233–239.
60. Brown, D. A., Kang, S. H., Gryaznov, S. M., et al. (1994) Effect of phosphorothioate modification of oligodeoxynucleotides on specific protein binding. *J. Biol. Chem.* **269**, 26,801–26,805.
61. Khaled, Z., Benimetskaya, L., Zeltser, R., et al. (1996) Multiple mechanisms may contribute to the cellular anti-adhesive effects of phosphorothioate oligodeoxynucleotides. *Nucleic Acids Res.* **24**, 737–745.
62. Hartmann, G., Krug, A., Waller-Fontaine, K., and Endres, S. (1996) Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: dependence on phosphorothioate modification and reversal by heparin. *Mol. Med.* **2**, 429–438.
63. Monteith, D. K., Henry, S. P., Howard, R. B., et al. (1997) Immune stimulation—a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Des.* **12**, 421–432.
64. Henry, S., Stecker, K., Brooks, D., Monteith, D., Conklin, B., and Bennett, C. F. (2000) Chemically modified oligonucleotides exhibit decreased immune stimulation in mice. *J. Pharmacol. Exp. Ther.* **292**, 468–479.
65. Tam, R. C., Wu-Pong, S., Pai, B., et al. (1999) Increased potency of an aptameric G-rich oligonucleotide is associated with novel functional properties of phosphorothioate linkages. *Antisense Nucleic Acid Drug Dev.* **9**, 289–300.
66. Krieg, A. M., Wu, T., Weeratna, R., et al. (1998) Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. USA* **95**, 12,631–12,636.
67. Stunz, L. L., Lenert, P., Peckham, D. W., Yi, A. K., Haxhinasto, S., Chang, M., Krieg, A. M., and Ashman, R. F. (2002) Inhibitory CpG oligonucleotides specifically block effects of stimulatory CpG oligonucleotides in B cells. *Eur. J. Immunol.* **32**, 1212–1222.
68. Lenert, P., Stunz, L. L., Yi, A. K., Krieg, A. M., and Ashman, R. F. (2001) CpG Stimulation of Primary Mouse B Cells is Blocked by Inhibitory Oligodeoxyribonucleotides at a Site Proximal to NF- κ B Activation. *Antisense Nucleic Acid Drug Dev.* **11**, 247–256.
69. Sester, D. P., Beasley, S. J., Sweet, M. J., et al. (1999) Bacterial/CpG DNA down-modulates colony stimulating factor-1 receptor surface expression on murine bone marrow-derived macrophages with concomitant growth arrest and factor-independent survival. *J. Immunol.* **163**, 6541–6550.
70. Kimura, Y., Sonehara, K., Kuramoto, E., et al. (1994) Binding of oligoguanylate to scavenger receptors is required for oligonucleotides to augment NK cell activity and induce IFN. *J. Biochem. (Tokyo)*. **116**, 991–994.

71. Pisetsky, D. S. and Reich, C. F., III (1998) The influence of base sequence on the immunological properties of defined oligonucleotides. *Immunopharmacology* **40**, 199–208.
72. Krug, A., Rothenfusser, S., Hornung, V., et al. (2001) Identification of CpG oligonucleotide sequences with high induction of IFN- α / β in plasmacytoid dendritic cells. *Eur. J. Immunol.* **31**, 2154–2163.
73. Lerner, R. A., Meinke, W., and Goldstein, D. A. (1971) Membrane-associated DNA in the cytoplasm of diploid human lymphocytes. *Proc. Natl. Acad. Sci. USA* **68**, 1212–1216.
74. Aggarwal, S. K., Wagner, R. W., McAllister, P. K., and Rosenberg, B. (1975) Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum-pyrimidine complexes by electron microscopy. *Proc. Natl. Acad. Sci. USA* **72**, 928–932.
75. Bennett, R. M., Gabor, G. T., and Merritt, M. M. (1985) DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. *J. Clin. Invest.* **76**, 2182–2190.
76. Bennett, R. M. (1993) As nature intended? The uptake of DNA and oligonucleotides by eukaryotic cells. *Antisense Res. Dev.* **3**, 235–241.
77. Hefeneider, S. H., McCoy, S. L., Morton, J. I., et al. (1992) DNA binding to mouse cells is mediated by cell-surface molecules: the role of these DNA-binding molecules as target antigens in murine lupus. *Lupus* **1**, 167–173.
78. Jacob, L., Lety, M. A., Choquette, D., et al. (1987) Presence of antibodies against a cell-surface protein, cross-reactive with DNA, in systemic lupus erythematosus: a marker of the disease. *Proc. Natl. Acad. Sci. USA* **84**, 2956–2959.
79. Bennett, C. F., Chiang, M. Y., Chan, H., Shoemaker, J. E., and Mirabelli, C. K. (1992) Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* **41**, 1023–1033.
80. Loke, S. L., Stein, C. A., Zhang, X. H., et al. (1989) Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. US* **86**, 3474–3478.
81. Yakubov, L. A., Deeva, E. A., Zarytova, V. F., et al. (1989) Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? *Proc. Natl. Acad. Sci. USA* **86**, 6454–6458.
82. Benimetskaya, L., Tonkinson, J. L., Koziolkiewicz, M., Karwowski, B., Guga, P., Zeltser, R., Stec, W., and Stein, C. A. (1995) Binding of phosphorothioate oligodeoxynucleotides to basic fibroblast growth factor, recombinant soluble CD4, laminin and fibronectin is P- chirality independent. *Nucleic Acids Res.* **23**, 4239–4245.
83. Rykova, E. Y., Laktionov, P. P., and Vlassov, V. V. (1999) Activation of spleen lymphocytes by plasmid DNA. *Vaccine* **17**, 1193–1200.
84. Emlen, W., Holers, V. M., Arend, W. P., and Kotzin, B. (1992) Regulation of nuclear antigen expression on the cell surface of human monocytes. *J. Immunol.* **148**, 3042–3048.
85. Yamamoto, T., Yamamoto, S., Kataoka, T., and Tokunaga, T. (1994) Lipofection of synthetic oligodeoxyribonucleotide having a palindromic sequence of AACGTT to murine splenocytes enhances interferon production and natural killer activity. *Microbiol. Immunol.* **38**, 831–836.

86. Manzel, L. and Macfarlane, D. E. (1999) Lack of immune stimulation by immobilized CpG-oligodeoxynucleotide. *Antisense Nucleic Acid Drug Dev.* **9**, 459–464.
87. Hanss, B., Leal-Pinto, E., Bruggeman, L. A., Copeland, T. D., and Klotman, P. E. (1998) Identification and characterization of a cell membrane nucleic acid channel. *Proc. Natl. Acad. Sci. USA* **95**, 1921–1926.
88. Krieg, A. M. (1995) Uptake and localization of phosphodiester and chimeric oligodeoxynucleotides in normal and leukemic primary cells. In: *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, S. Akhtar (ed.), CRC Press, Boca Raton, FL, pp. 177–190.
89. Hanss, B., Stein, C. A., and Klotman, P. E. (1998) Cellular uptake and biodistribution of oligodeoxynucleotides. 111–127.
90. Beltinger, C., Saragovi, H. U., Smith, R. M., et al. (1995) Binding, uptake, and intracellular trafficking of phosphorothioate- modified oligodeoxynucleotides. *J. Clin. Invest.* **95**, 1814–1823.
91. Krieg, A. M., Tonkinson, J., Matson, S., et al. (1993) Modification of antisense phosphodiester oligodeoxynucleotides by a 5' cholesteryl moiety increases cellular association and improves efficacy. *Proc. Natl. Acad. Sci. USA* **90**, 1048–1052.
92. Krieg, A. M., Gmelig-Meyling, F., Gourley, M. F., Kisch, W. J., Chrisey, L. A., and Steinberg, A. D. (1991) Uptake of oligodeoxyribonucleotides by lymphoid cells is heterogeneous and inducible. *Antisense Res. Dev.* **1**, 161–171.
93. Zhao, Q., Song, X., Waldschmidt, T., Fisher, E., and Krieg, A. M. (1996) Oligonucleotide uptake in human hematopoietic cells is increased in leukemia and is related to cellular activation. *Blood*. **88**, 1788–1795.
94. Kronenwett, R., Steidl, U., Kirsch, M., Sczakiel, G., and Haas, R. (1998) Oligodeoxyribonucleotide uptake in primary human hematopoietic cells is enhanced by cationic lipids and depends on the hematopoietic cell subset. *Blood*. **91**, 852–862.
95. Hartmann, G., Krug, A., Bidlingmaier, M., Hacker, U., Eigler, A., Albrecht, R., Strasburger, C. J., and Endres, S. (1998) Spontaneous and cationic lipid-mediated uptake of antisense oligonucleotides in human monocytes and lymphocytes. *J. Pharmacol. Exp. Ther.* **285**, 920–928.
96. Nicklin, P. S., Craig, S. J., and Phillips, J. A. (1998) Pharmacokinetic Properties of Phosphorothioates in Animals - Absorption, Distribution, Metabolism and Elimination. In: *Antisense Research and Application*, S. T. Crooke (ed.), Springer-Verlag, Heidelberg, Germany, pp. 141–168.
97. Zhao, Q., Zhou, R., Tamsamani, J., Zhang, Z., Roskey, A., and Agrawal, S. (1998) Cellular distribution of phosphorothioate oligonucleotide following intravenous administration in mice. *Antisense Nucleic Acid Drug Dev.* **8**, 451–458.
98. Yi, A. K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J., and Krieg, A. M. (1998) CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* **160**, 4755–4761.

99. Macfarlane, D. E. and Manzel, L. (1998) Antagonism of immunostimulatory CpG-oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. *J. Immunol.* **160**, 1122–1131.
100. Streckowski, L., Zegrocka, O., Henary, M., et al. (1999) Structure-activity relationship analysis of substituted 4-quinolinamines, antagonists of immunostimulatory CpG-oligodeoxynucleotides. *Bioorg. Med. Chem. Lett.* **9**, 1819–1824.
101. Hacker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.
102. Yi, A. K. and Krieg, A. M. (1998) Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493–4497.
103. Hacker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., and Wagner, H. (2000) Immune Cell Activation by Bacterial CpG-DNA through Myeloid Differentiation Marker 88 and Tumor Necrosis Factor Receptor-Associated Factor (TRAF)6. *J. Exp. Med.* **192**, 595–600.
104. Schnare, M., Holtdagger, A. C., Takeda, K., Akira, S., and Medzhitov, R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* **10**, 1139–1142.
105. Bauer, S., Kirschning, C. J., Hacker, H., et al. (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc. Natl. Acad. Sci. USA* **98**, 9237–9242.
106. Chuang, T. H. and Ulevitch, R. J. (2000) Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur. Cytokine Netw.* **11**, 372–378.
107. Du, X., Poltorak, A., Wei, Y., and Beutler, B. (2000) Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur. Cytokine Netw.* **11**, 362–371.
108. Hacker, H., Mischak, H., Hacker, G., Eser, S., Prenzel, N., Ullrich, A., and Wagner, H. (1999) Cell type-specific activation of mitogen-activated protein kinases by CpG-DNA controls interleukin-12 release from antigen-presenting cells. *EMBO J.* **18**, 6973–6982.
- 108a. Yi, A. K., Yoon, J. G., Yeo, S. J., et al. (2002) Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th 1 response. *J. Immunol.* **168**, 4711–4720.
109. Kistler, B., Rolink, A., Marienfeld, R., Neumann, M., and Wirth, T. (1998) Induction of nuclear factor- κ B during primary B cell differentiation. *J. Immunol.* **160**, 2308–2317.
110. Sha, W. C. (1998) Regulation of immune responses by NF- κ B/Rel transcription factor [published erratum appears in J Exp Med 1998 Feb 16;187(4):661]. *J. Exp. Med.* **187**, 143–146.
111. McIntyre, K. W., Lombard-Gillooly, K., Perez, J. R., et al. (1993) A sense phosphorothioate oligonucleotide directed to the initiation codon of transcrip-

- tion factor NF- κ B p65 causes sequence-specific immune stimulation. *Antisense Res. Dev.* **3**, 309–322.
112. Stacey, K. J., Sweet, M. J., and Hume, D. A. (1996) Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116–2122.
 113. Sparwasser, T., Miethke, T., Lipford, G., Erdmann, A., Hacker, H., Heeg, K., and Wagner, H. (1997) Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor- α -mediated shock. *Eur. J. Immunol.* **27**, 1671–1679.
 114. Sen, C. K. and Packer, L. (1996) Antioxidant and redox regulation of gene transcription [see comments]. *FASEB J.* **10**, 709–720.
 115. Muller, J. M., Rupec, R. A., and Baeuerle, P. A. (1997) Study of gene regulation by NF- κ B and AP-1 in response to reactive oxygen intermediates. *Meth-ods.* **11**, 301–312.
 116. Gupta, R. K. and Siber, G. R. (1995) Adjuvants for human vaccines—current status, problems and future prospects. *Vaccine* **13**, 1263–1276.
 117. Bireland, M. L. and Monroe, J. G. (1997) Biochemistry of antigen receptor signaling in mature and developing B lymphocytes. *Crit. Rev. Immunol.* **17**, 353–385.
 118. DeFranco, A. L. (1997) The complexity of signaling pathways activated by the BCR. *Curr. Opin. Immunol.* **9**, 296–308.
 119. Sweet, M. J., Stacey, K. J., Ross, I. L., Ostrowski, M. C., and Hume, D. A. (1998) Involvement of Ets, rel and Sp1-like proteins in lipopolysaccharide-mediated activation of the HIV-1 LTR in macrophages. *J. Inflamm.* **48**, 67–83.
 120. Sun, S., Zhang, X., Tough, D. F., and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* **188**, 2335–2342.
 121. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3049.
 122. Chace, J. H., Hooker, N. A., Mildenstein, K. L., Krieg, A. M., and Cowdery, J. S. (1997) Bacterial DNA-induced NK cell IFN- γ production is dependent on macrophage secretion of IL-12. *Clin. Immunol. Immunopathol.* **84**, 185–193.
 123. Schwartz, D. A., Quinn, T. J., Thorne, P. S., Sayeed, S., Yi, A. K., and Krieg, A. M. (1997) CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* **100**, 68–73.
 124. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants [see comments]. *Nat. Med.* **3**, 849–854.
 125. Zhao, Q., Tamsamani, J., Zhou, R. Z., and Agrawal, S. (1997) Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev.* **7**, 495–502.
 126. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.

127. Boggs, R. T., McGraw, K., Condon, T., et al. (1997) Characterization and modulation of immune stimulation by modified oligonucleotides. *Antisense Nucleic Acid Drug Dev.* **7**, 461–471.
128. Sester, D. P., Naik, S., Beasley, S. J., Hume, D. A., and Stacey, K. J. (2000) Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. *J. Immunol.* **165**, 4165–4173.
129. Lipford, G. B., Bendigs, S., Heeg, K., and Wagner, H. (2000) Poly-guanosine motifs costimulate antigen-reactive CD8 T cells while bacterial CpG-DNA affect T-cell activation via antigen-presenting cell-derived cytokines. *Immunology* **101**, 46–52.
130. Bishop, J. S., Guy-Caffey, J. K., Ojwang, J. O., et al. (1996) Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. *J. Biol. Chem.* **271**, 5698–5703.
131. Lang, R., Hultner, L., Lipford, G. B., Wagner, H., and Heeg, K. (1999) Guanosine-rich oligodeoxynucleotides induce proliferation of macrophage progenitors in cultures of murine bone marrow cells. *Eur. J. Immunol.* **29**, 3496–3506.

IFN-Dependent Pathways for Stimulation of Memory CD8⁺ Cells

Jonathan Sprent

1. INTRODUCTION

In young life, most typical T cells expressing $\alpha\beta$ T-cell receptors (TCR) are naive resting cells and comprise a mixture of CD4⁺ and CD8⁺ cells (1,2). These T cells rarely divide in normal unimmunized animals but mount intense TCR-dependent proliferative responses when confronted with specific foreign ligands, i.e., foreign peptides bound to major histocompatibility complex (MHC) molecules on specialized antigen-presenting cells (APC). After eliminating the pathogen concerned, a small proportion of the proliferating T cells survives and forms long-lived memory cells (3–5). These cells are phenotypically distinct from naive T cells. For example, naive T cells express a low density of surface CD44 molecules (CD44^{lo}) whereas memory cells are CD44^{hi}. T cells with a CD44^{hi} memory phenotype comprise only 10–20% of total T cells in young animals but become a predominant population in old age. These “memory-phenotype” cells are considered to be the progeny of naive T cells responding to a variety of environmental antigens.

A notable feature of memory-phenotype T cells is that these cells divide much more rapidly than naive-phenotype cells (6). This article reviews recent evidence on the stimuli controlling proliferation of memory-phenotype cells. Special emphasis is given to the capacity of products of microorganisms, including CpG DNA, to augment proliferation of the CD8⁺ subset of memory-phenotype cells.

2. RESULTS AND DISCUSSION

2.1. *Background Proliferation and the Role of the MHC*

For naive T cells in mice, it has recently become apparent that the long-term survival of these cells requires continuous contact with MHC molecules (5,7,8). Thus, although typical naive T cells can survive for several months in normal hosts, naive T cells tend to disappear over a period of a few weeks after transfer to MHC⁻ hosts, i.e., when CD4⁺ cells are transferred to MHC II⁻ mice or CD8⁺ cells are transferred to MHC I⁻ hosts. These findings have given rise to the notion that the longevity of naive T cells requires low-level TCR signaling through contact with MHC-associated self peptides.

In marked contrast to naive cells, contact with MHC ligands does not appear to be required for the survival of memory-phenotype T cells. Thus, contrary to an initial report (7), several groups have found that memory-phenotype CD44^{hi} T cells survive for prolonged periods after transfer to MHC⁻ hosts (9,10). Moreover, at least for CD8⁺ cells, the high turnover (proliferation rate) of memory-phenotype T cells is maintained in MHC⁻ hosts (9).

The important conclusion from these findings is that the high turnover of memory-phenotype T cells is MHC-independent. What then is the stimulus for proliferation of these cells? In considering this question it is relevant to discuss the evidence that the turnover of memory-phenotype T cells is increased following exposure to products of microorganisms.

2.2. *Bystander Stimulation of Memory-Phenotype T Cells*

Proliferation of T cells in vivo can be studied by administering the DNA precursor, bromodeoxyuridine (BrdU), in the drinking water followed by staining T cells for BrdU incorporation (6). With this technique we documented intense proliferation of T cells during viral infections and concluded that much of the proliferation was nonantigen specific (11). This conclusion turned out to be largely incorrect because the subsequent development of highly-sensitive methods for detecting antigen-specific T cells showed that most of the T cells proliferating during viral infections are responding to specific MHC-bound viral peptides (12,13). Nevertheless, a recent re-evaluation of this issue has shown that nonantigen-specific "bystander" proliferation is indeed apparent in viral infections, though only during the early stages of infection (14); thereafter, the response is dominated by antigen-specific T cells.

Because viral infections are associated with conspicuous production of interferons (IFNs), especially type I IFN (IFN-I), we considered the possibility that bystander proliferation of T cells could be mediated by IFN-I. In favor of this idea, injecting mice with purified IFN-I or Poly I:C, a strong

stimulator of IFN-I synthesis, induced a sharp, transient increase in the rate of T-cell proliferation (11). This proliferative response affected memory-phenotype (CD44^{hi}) T cells, including both CD4⁺ and CD8⁺ cells; stimulation of naive-phenotype cells was minimal. The possibility that IFN-I-induced proliferation of CD44^{hi} CD8⁺ cells required TCR ligation was excluded by the finding that proliferation occurred when MHC I⁻ (β2m⁻) CD8⁺ cells (raised in bone marrow chimeras) were exposed to Poly I:C in MHC I⁻ hosts (11). Based on this finding, it was concluded that the T cells were stimulated via a TCR-independent bystander mechanism. Significantly, for Poly I:C injection, the proliferative response was minimal in IFN-IR⁻ hosts, indicating that the response was IFN-I dependent (11).

Subsequent studies showed that bystander proliferation of CD44^{hi} CD8⁺ cells in vivo could be induced by various products of microorganisms including lipopolysaccharide (LPS), CpG DNA and also by killed gram⁺ and gram⁻ bacteria (15,16). Based on studies with IFN-IR⁻ mice, bystander proliferation elicited by these agents was IFN-I dependent. In the case of CpG DNA, strong bystander proliferation occurred following injection of insect (*Drosophila*) DNA or synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs.

In addition to IFN-I and the IFN-I-inducing agents discussed previously, we have observed comparable bystander proliferation of CD44^{hi} CD8⁺ cells after injection of IFN-γ and also IL-12 and IL-18 (D. Tough, X. Zhang and J. Sprent, submitted for publication). For these three cytokines, proliferation is abolished in IFN-γ⁻ mice, indicating dependence on IFN-γ.

Collectively, the above findings indicate that bystander proliferation of CD44^{hi} CD8⁺ cells involves two separate pathways, one controlled by IFN-I and the other by IFN-γ.

2.3. Mechanism of Bystander Stimulation by IFNs

Initially we assumed that IFNs stimulate T cells directly through binding to IFNR. This idea turned out to be incorrect, however, because adding either IFN-I or IFN-γ to purified T cells in vitro fails to cause proliferation (17). In fact IFN-I has a marked antiproliferative effect on T cells (18). Interestingly, IFN-I and to a lesser extent IFN-γ do cause partial activation of T cells. Thus, adding IFN-I to purified T cells in vitro causes upregulation of a variety of surface markers, including CD69, B7-2, MHC I, Ly6C, and ICAM-1 (18); by contrast, partial activation of T cells by IFN-γ is limited to upregulation of ICAM-1. However neither cytokine causes entry into cell cycle.

In view of these findings, we reasoned that proliferation of CD44^{hi} CD8⁺ cells in vivo elicited by IFNs and IFN-inducing agents is probably mediated through synthesis of a secondary cytokine, i.e., an effector cytokine that acts

directly on T cells. As discussed in Subheading 2.4. there is now strong evidence that the effector cytokine is IL-15.

2.4. Role of IL-15 in Bystander Proliferation

IL-15 is an IL-2-like cytokine which is synthesized by a wide variety of cell types, with the notable exception of T cells (19). The authors considered IL-15 a likely candidate for being the effector cytokine for bystander proliferation for three reasons (17). First, IFNs and IFN inducers induced strong synthesis of IL-15 mRNA by purified macrophages in vitro. Second, IL-2R β (CD122), an important receptor for IL-15 (and IL-2) was found to be expressed at a much higher level on CD44^{hi} CD8⁺ cells (the main targets for bystander proliferation) than on CD44^{hi} CD4⁺ cells or on naive T cells. Third, unlike IFNs, IL-15 induced strong and selective proliferation of purified CD8⁺ cells in vitro. Corroborating these findings, other workers reported that IL-15 transgenic mice show a selective expansion of CD44^{hi} CD8⁺ cells (20).

More recently the authors have obtained direct support for the notion that bystander proliferation is mediated through production of IL-15. The key finding is that bystander proliferation induced by Poly I:C after adoptive transfer of T cells is virtually abolished in IL-15⁻ hosts (A. Judge, X. Zhang and J. Sprent, unpublished data).

2.5. IL-15 and T-Cell Survival

The observation that IL-15 controls bystander proliferation of CD44^{hi} CD8⁺ cells elicited by IFNs raises the question whether IL-15 plays a role in controlling the steady-state survival and turnover of CD44^{hi} CD8⁺ cells in normal unimmunized mice. In favor of this possibility IL-15R α ⁻ (21) and IL-15⁻ (22) mice both display a selective paucity of CD44^{hi} CD8⁺ cells; these mice show no reduction in numbers of CD44^{hi} CD4⁺ cells or naive T cells. In addition, injecting normal mice with anti-IL-2R β mAb is reported to reduce the background turnover of CD44^{hi} CD8⁺ cells (23). Interestingly, injection of anti-IL-2 mAb has the opposite effect and augments background proliferation.

Collectively, these findings indicate that IL-15 does play a critical role in controlling both the survival and turnover of CD44^{hi} CD8⁺ cells in normal animals. By contrast, at least for proliferation, IL-2 appears to inhibit the positive function of IL-15 (23,24). Thus, even though IL-15 and IL-2 both bind to IL-2R β and signal through the γ c chain, these two cytokines seem to elicit quite separate patterns of intracellular signaling. The molecular basis of this difference is still unclear.

3. CONCLUDING COMMENTS

As outlined above, IL-15 plays a key role in maintaining the survival of memory-phenotype CD8⁺ cells. In normal unimmunized animals, continu-

ous low-level stimulation of CD44^{hi} CD8⁺ cells through background release of IL-15 promotes the long-term survival of these cells and also causes intermittent entry into cell cycle. Through mechanisms that are still poorly understood, the stimulatory effects of IL-15 are countered by the action of IL-2. Such homeostatic control by IL-15 and IL-2 presumably serves to maintain total numbers of memory-phenotype T cells at a near-constant level, life and death of the cells being kept at an equilibrium. This scenario poses a number of questions, including the issue of how the background level of IL-15 (and IL-2) is maintained. Here, the simplest possibility is that IL-15 is released constitutively by various stromal cells. Alternatively, background levels of IL-15 may reflect continuous stimulation of non-T cells by IFNs and perhaps other cytokines. This question could be addressed by studying IL-15 levels in IFN⁻ mice.

The capacity of IFNs and IFN-inducing agents to stimulate IL-15 production explains the burst of bystander CD44^{hi} CD8⁺ cell proliferation seen in viral infections. As discussed earlier, bystander proliferation is apparent in the early stages of viral infections—presumably reflecting local release of IFNs—but is then overwhelmed by proliferation of antigen-specific T cells. The fate of T cells subjected to bystander proliferation requires comment. In our initial studies we found that the progeny of T cells proliferating in response to Poly I:C survived for prolonged periods (11). In light of this finding we suggested that bystander proliferation could be beneficial in keeping memory CD8⁺ cells alive. Although recent data on the prosurvival effects of IL-15 are consistent with this idea (see above), other workers have reported that bystander proliferation can have the opposite effect and lead to attrition of memory cells (25,26). A possible explanation for this discrepancy is that one of the important inducers of bystander stimulation, namely IFN-I, can have a marked antiproliferative effect on T-cell proliferation, including proliferation elicited by IL-15 (18). Hence, during viral infections, the fate of bystander memory cells (memory cells specific for other antigens) may hinge on the relative concentrations of several different cytokines, notably IL-15, IL-2, IFN-I, and IFN- γ , each of these cytokines exerting different effects on memory cells. During infections of moderate intensity, IL-15 production elicited by IFNs may serve to boost the survival of “third-party” memory CD8⁺ cells and prevent these cells from being overwhelmed by the massive clonal expansion of virus-specific T cells. Conversely, in overwhelming viral infections, very high levels of IFN-I may exert an antiproliferative effect on T cells, thus reducing bystander proliferation elicited by IL-15. In addition, such inhibition could be accentuated by parallel synthesis of toxic cytokines such as TNF- α and stress associated with release of corticosteroids, thereby leading to cell injury and death.

REFERENCES

1. Sprent, J. (1993) T lymphocytes and the thymus, in *Fundamental Immunology*, 3rd Edition, (Paul, W. E., ed.), Raven Press, New York, pp. 75–110.
2. Benoist, C. and Mathis, D. (1999) T-lymphocyte differentiation and biology, in *Fundamental Immunology*, 4th Edition, (Paul, W. E., ed.), Lippincott-Raven, Philadelphia, pp. 367–409.
3. Sprent, J. (1993) Lifespans of naive, memory and effector lymphocytes. *Curr. Opin. Immunol.* **5**, 433–438.
4. Dutton, R. W., Bradley, L. M., and Swain, S. L. (1998) T cell memory. *Annu. Rev. Immunol.* **16**, 201–223.
5. Sprent, J. and Surh, C. D. (2000) Generation and maintenance of memory cells. *Curr. Opin. Immunol.*, In press.
6. Tough, D. F. and Sprent, J. (1994) Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* **179**, 1127–1135.
7. Tanchot, C., Lemonnier, F. A., Perarnau, B., Freitas, A. A., and Rocha, B. (1997) Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* **276**, 2057–2062.
8. Rooke, R., Waltzinger, C., Benoist, C., and Mathis, D. (1997) Targeted complementation of MHC class II deficiency by intrathymic delivery of recombinant adenoviruses. *Immunity* **7**, 123–134.
9. Murali-Krishna, K., Lau, L. L., Sambhara, S., Lemonnier, F., Altman, J., and Ahmed, R. (1999) Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377–1381.
10. Swain, S. L., Hu, H. and Huston, G. (1999) Class II-independent generation of CD4 memory T cells from effectors. *Science* **286**, 1381–1383.
11. Tough, D. F., Borrow, P. and Sprent, J. (1996) Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science* **272**, 1947–1950.
12. Murali-Krishna, K., Altman, J. D., Suresh, M., et al. (1998) Counting antigen-specific CD8 T cells: A reevaluation of bystander activation during viral infection. *Immunity* **8**, 177–188.
13. Butz, E. A. and Bevan, M. J. (1998) Massive expansion of antigen-specific CD8⁺ T cells during an acute virus infection. *Immunity* **8**, 167–176.
14. Andreasen, S. O., Christensen, J. P., Marker, O., and Thomsen, A. R. (1999) Virus-induced non-specific signals cause cell cycle progression of primed CD8(+) T cells but do not induce cell differentiation. *Int. Immunol.* **11**, 1463–1473.
15. Tough, D. F., Sun, S., and Sprent, J. (1997) T cell stimulation in vivo by lipopolysaccharide (LPS). *J. Exp. Med.* **185**, 2089–2094.
16. Sprent, J., Zhang, X., Sun, S., and Tough, D. (2000) T cell proliferation in vivo and the role of cytokines. *Phil. Trans. R. Soc. Lond. Biol. Sci.* **355**, 317–322.
17. Zhang, X., Sun, S., Hwang, I., Tough, D. F., and Sprent, J. (1998) Potent and selective stimulation of memory-phenotype CD8⁺ T cells in vivo by IL-15. *Immunity* **8**, 591–599.
18. Sun, S., Zhang, X., Tough, D. F., and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* **188**, 2335–2342.

19. Waldmann, T. A. and Tagaya, Y. (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. *Annu. Rev. Immunol.* **17**, 19–49.
20. Nishimura, H., Yajima, T., Naiki, Y., et al. (2000) Differential roles of interleukin 15 mRNA isoforms generated by alternative splicing in immune responses in vivo. *J. Exp. Med.* **191**, 157–170.
21. Lodolce, J. P., Boone, D. L., Chai, S., et al. (1998) IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9**, 669–676.
22. Kennedy, M. K., Glaccum, M., Brown, S. N., et al. (2000) Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* **191**, 771–780.
23. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J., and Marrack, P. (2000) Control of homeostasis of CD8⁺ memory T cells by opposing cytokines. *Science* **288**, 675–678.
24. Dai, Z., Konieczny, B. T. and Lakkis, F. G. (2000) The dual role of IL-2 in the generation and maintenance of CD8⁺ memory T cells. *J. Immunol.* **165**, 3031–3036.
25. Selin, L. K., Lin, M. Y., Kraemer, K. A., Pardoll, D. M., Schneck, J. P., Varga, S. M., Santolucito, P. A., Pinto, A. K. and Welsh, R. M. (1999) Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity* **11**, 733–742.
26. Busch, D. H., Kerksiek, K. M. and Pamer, E. G. (2000) Differing roles of inflammation and antigen in T cell proliferation and memory generation. *J. Immunol.* **164**, 4063–4070.

Cross-Priming of CD8⁺ T Cells by Immunostimulatory Sequence DNA

Hearn Jay Cho, Sandip Datta,
and Eyal Raz

1. INTRODUCTION

Microbial DNA containing unmethylated CpG dinucleotides (Immunostimulatory sequence [ISS] DNA, or CpG motifs) act upon cells of the innate immune system such as phagocytes (macrophages and dendritic cells [DC]) and NK cells and upon B cells, promoting the expression of pro-inflammatory cytokines and surface molecules (1–4). They have little direct effect upon CD8⁺ and CD4⁺ T cells, yet animal models have shown that ISS-based vaccines promote two antigen-specific T cell responses: cytotoxic lymphocyte (CTL) activity and a T_h1-type helper phenotype (5–8) (Fig. 1). This phenomenon has been observed with plasmid DNA vaccines (9), protein and synthetic ISS oligodeoxynucleotide (ISS-ODN) co-administration (6, 10–12), and protein-ISS-ODN conjugate vaccines (13, 14). Similar T-cell responses have been shown with distinct experimental antigens, including bacterial β -galactosidase (6), chicken ovalbumin (13), hepatitis B virus surface antigen (15), and human immunodeficiency virus gp120 (14). Effective CTL and T_h1 priming has been observed with both intradermal (13, 16) and intranasal (12) routes of immunization. Of note, priming of CTL activity with ISS-ODN and protein antigen, either coadministration or as conjugates, is independent of major histocompatibility complex (MHC) class II-restricted T helper activity, whereas priming by plasmid DNA vaccines is not (13). These findings suggest that ISS-ODN act upon antigen-presenting cells (APC), particularly dendritic cells (DC), allowing them to directly prime CTL against exogenous antigens in the absence of T-cell help.

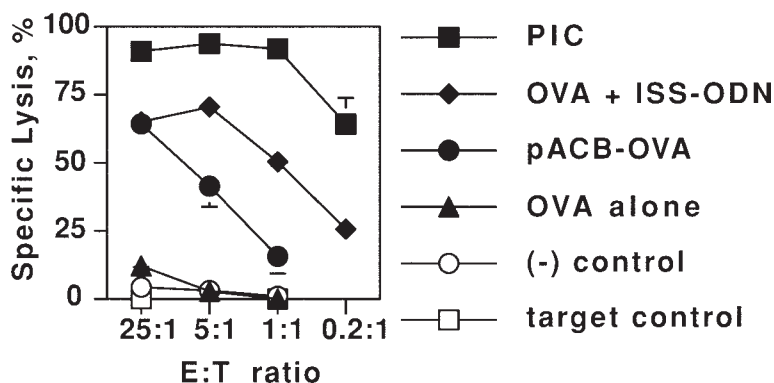


Fig. 1. Priming of antigen-specific cytotoxic lymphocyte activity by ISS-based vaccines. Wt mice were immunized on day zero and 14 with experimental and control vaccines formulated with ovalbumin (OVA) and ISS-ODN, and total splenocytes recovered at six weeks and evaluated by secondary CTL assays. Protein-ISS conjugate (PIC, ■) primed very high levels of OVA-specific CTL activity compared to OVA and ISS-ODN co-administration (◆), plasmid DNA pACB-OVA (●), or OVA alone (▲). Splenocytes from mice vaccinated with PIC did not lyse target cells loaded with an irrelevant influenza virus-derived antigenic peptide (target control, ○). Adapted from reference (13).

What are the mechanisms by which ISS-based vaccines prime antigen-specific CTL activity?

Priming of CTL requires two signals. Signal one is an antigenic peptide displayed in the context of MHC class I for recognition by the CTL T-cell receptor, and signal two is appropriate costimulatory molecules, either on the cell surface for cognate cell-to-cell interaction or soluble factors such as cytokines (17–19). Analysis of CTL activation by ISS-based vaccines has been greatly aided by recent advances in understanding the phenomenon of cross-priming, the priming of CTL activity against exogenous antigens. This is the major mechanism of priming CTL against intracellular pathogens such as viruses. Current models of cross-priming consist of two steps: a CD40/CD40 ligand (CD40L)-dependent “licensing” interaction between APC and T_H , followed by an activating interaction between “licensed” APC and CTL (Fig. 2) (20–23). This sequence can be temporally and spatially dissociated. The “licensing” model of cross-priming implies that APC “cross-present” exogenous antigens. Cross-presentation is the display of antigenic peptides from exogenous sources in the context of MHC class I for recognition by the CTL T-cell receptor. In addition, the “licensing” interaction presumably influences the expression of costimulatory molecules by the APC, resulting

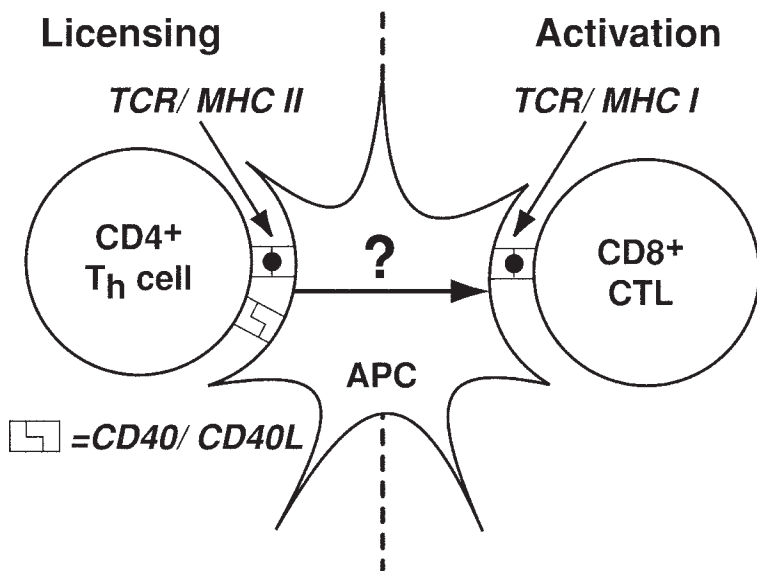


Fig. 2. Licensing model of cross-priming. Antigen presenting cells (APC) act as a “conditioned bridge” between CD4⁺ T helper and CD8⁺ cytotoxic lymphocytes. In the licensing interaction, antigen is presented on MHC class II of the APC to the T_h T cell receptor (TCR). Costimulatory engagement of the CD40 receptor on APC and CD40 L (CD154) on the T_h cell results in “licensing” of the APC, rendering them competent to proceed to the activation step. “Licensed” APC can then activate naïve CD8⁺ that recognize MHC class I-restricted antigens presented on the APC via the CTL TCR. The nature of the molecules that are regulated by the licensing step (denoted by “?” arrow) are discussed in the text. Adapted from reference (13).

in an APC-CTL interaction that promotes effective priming. The following discussion reviews current concepts in the action of ISS on cross-presentation and costimulation in cross-priming antigen-specific CTL activity.

2. RESULTS

2.1. ISS and Cross-Presentation

APC phagocytize exogenous antigens and degrade them in the endosomal compartment for display in the context of MHC class II. Class II-restricted antigen presentation is vital to the T helper/APC licensing step. It has become apparent, however, that APC also cross-present exogenous protein antigens in the context of MHC class I as well as class II, allowing recognition of these antigens by CTL T-cell receptors. Three mechanisms for this phenomenon have been described: a Transporter associated with Antigen

Presentation (TAP)-dependent pathway, also known as endosome-to-cytosol shuttling (24,25), a TAP-independent, or “regurgitant,” pathway (26,27), and a “recycling” pathway (28). Animal models using engineered viruses targeted to myeloid or non-myeloid tissue have elegantly demonstrated that TAP-dependent endosome-to-cytosol shuttling is required to generate anti-viral CTL *in vivo* (29). The *in vivo* significance of the TAP-independent pathways is unknown, although CTL can be primed against certain viral antigens in a TAP-independent manner (30).

Although there is no definitive evidence that ISS directly affects cross-presentation, there is a body of data that suggests ISS may facilitate cross-presentation at several levels. As previously noted, ISS stimulation increases surface expression of MHC class I and II, which may provide greater antigenic density on APC (4). In addition to increases surface presentation of antigen, ISS also appears to affect one of the intracellular mechanism of cross-presentation.

TAP activity appears to be required for priming of CTL by ISS-based vaccines. Immunization experiments were conducted with ISS coadministered with an experimental antigen ovalbumin (OVA) into bone marrow chimeric mice. Bone marrow chimeras were constructed by reconstituting lethally-irradiated wild-type (WT) mice with bone marrow from either WT or TAP^{-/-} syngeneic mice. Chimeras were used because TAP^{-/-} mice do not support the development of CD8⁺ CTL (31). These experiments showed that WT→WT chimeras primed OVA-specific CTL activity at levels similar to WT mice in response to immunization with ISS and OVA, but TAP^{-/-}→WT chimeras did not exhibit any detectable OVA-specific CTL activity (Fig. 3a) (32). This finding strongly suggests that TAP-dependent endosome-to-cytosol shuttling is required for CTL priming by ISS-based vaccines.

Does ISS stimulation have any direct effect upon TAP activity? To address this question, mouse bone marrow-derived macrophages (BM-DM) were stimulated *in vitro* with ISS-ODN, and cells were harvested at subsequent time points and their total RNA recovered. Reverse transcriptase-polymerase chain reaction (RT-PCR) with primers for both TAP1 and TAP2 (the subunits of TAP) showed that at rest, BM-DM transcribe very little message for TAP1 and 2. ISS-stimulation increased transcription of TAP1 and 2 detectable four hours after stimulation (Fig. 3B) (32). Similar results were seen *in vivo*. Wt mice were stimulated with intravenous ISS-ODN, and total splenocytes were harvested for RT-PCR analysis. Total splenocyte TAP expression increased within two hours of ISS stimulation, with persistent message detectable up to six hours after stimulation (Fig. 3C) (32). These

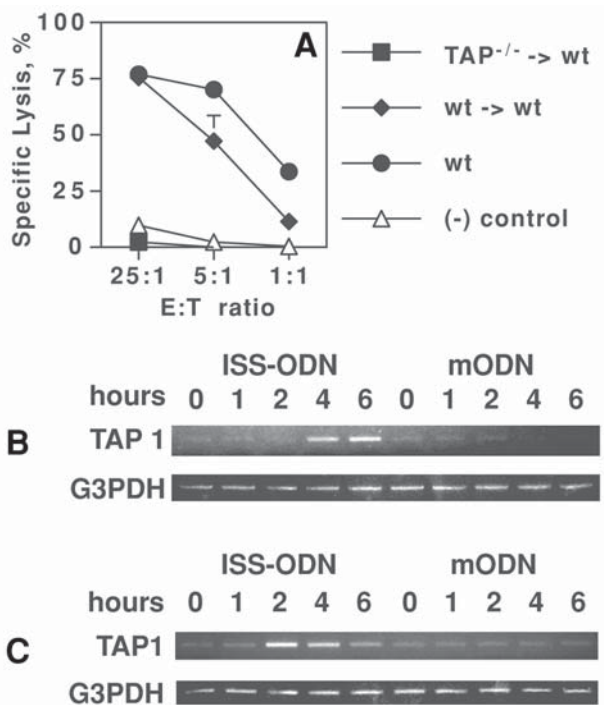


Fig. 3. TAP activity in cross-priming by ISS-based vaccines. **(A)** TAP activity is required for cross-priming by ISS-based vaccines. Wt→wt and TAP^{-/-}→wt bone marrow chimeras were immunized with OVA and ISS-ODN co-administration as described in Fig. 1. WT chimeras (◆) primed OVA-specific CTL similar to WT (●) mice, but TAP^{-/-} chimeras (■) did not prime CTL, indicating that TAP activity is required for cross-priming by ISS-based vaccines. **(B)** ISS-ODN stimulation induces TAP expression in vitro. WT bone marrow-derived macrophages were stimulated in culture with ISS-ODN and harvested at subsequent time points and analyzed by RT-PCR. RNA transcripts for TAP1 were detected at four and six hours poststimulation. RT-PCR of G3PDH transcripts are shown for comparison. Induction of TAP2 transcription followed similar kinetics (data not shown). **(C)** ISS-ODN stimulation induces TAP expression in vivo. WT mice were injected intravenously with ISS-ODN and total splenocytes were recovered at subsequent time points and analyzed by RT-PCR. TAP1 transcription is detectable at one hour, peaks at two hours, and this effect persists as long as six hours. TAP2 expression followed similar kinetics (data not shown). Adapted with permission from ref. 32

results suggest that ISS stimulation can increase expression of TAP in APC. Since CTL priming by ISS-based vaccines requires TAP, this regulatory effect of ISS may contribute to enhanced cross-presentation and subsequent CTL priming.

2.2. ISS and Costimulation

As noted earlier, the licensing step of cross-priming is dependent upon CD40/ CD40L interaction between APC and T_h cells, respectively (22,23). The activation of CTL in the absence of T-cell help strongly suggests that ISS can replace the licensing functions of the T_h -APC cell interaction. ISS stimulation upregulates proinflammatory cytokines and surface molecules, which may account for this effect (4,8). The requirements for the activation step are not fully elucidated, but several costimulatory molecules, including CD40, B7-1 and B7-2, ICAM-1, and IL-12, have been implicated in the activation of CTL (33–36).

The role of different costimulatory molecules in activation of CTL by ISS has been studied using gene-deficient mice and monoclonal antibody (mAb) blockade. Immunization of CD40^{-/-} mice or WT mice treated with mAb against CD40L with OVA and ISS-ODN coadministration results in similar levels of antigen-specific CTL activity as wt mice (Fig. 4A) (32). Interestingly, in the experimental animals, OVA-specific T_h1-biased helper cell activation, as measured by IFN γ secretion upon antigenic restimulation and OVA-specific IgG_{2a} titers, is dramatically decreased. This result shows that although CD40/CD40L signaling is required for licensing of APC and for T_h activation, the priming of CTL by APC that have been licensed by ISS stimulation is independent of CD40/ CD40L. In addition, APC function in this setting does not require intact CD40 receptor complexes.

In contrast, immunization of mice that have been treated with mAb blockade of B7-1 and -2 results in 52–80% reduction in CTL priming by OVA and ISS-ODN (Fig. 4B) (32). CD28 is a receptor for B7 molecules that is associated with activation CD4⁺ and CD8⁺ T cell (37). Immunization of CD28^{-/-} mice with OVA and ISS-ODN results in reduced OVA-specific CTL similar to mAb blockade. The observed decreases in CTL priming seen in anti-B7 mAb or CD28^{-/-} mice were not affected by the addition of mAb blockade against CD40L, supporting the findings in the CD40^{-/-} mice. These results show that B7/CD28 binding is the dominant costimulatory signal in the interaction between ISS-stimulated APC and naive CTL. Investigation with B7-1^{-/-} and B7-2^{-/-} double knockout mice immunized with inactivated tumor cells support the dominant role for B7/ CD28 interaction in cross-priming CTL, and suggest that the upregulation of B7 molecules on APC in vivo is dependent upon CD40/ CD40L licensing (38,39).

Immunization of IL-12 β ^{-/-} mice with OVA and ISS-ODN results in 35% less CTL activity when compared to WT (32). The addition of anti-B7 mAb blockade to IL-12 β ^{-/-} mice results in CTL activity similar to that seen in B7 blockade alone. These results indicate that although IL-12 contributes to

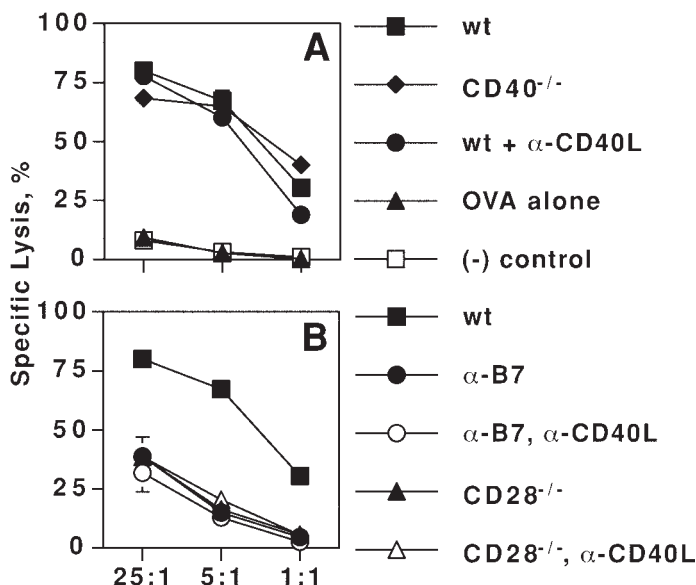


Fig. 4. Costimulation for cross-priming by ISS-based vaccines is mediated primarily by B7-1 and -2. (A) CD40/ CD40L does not participate in the activation step of cross-priming. Wt mice pre-treated with mAb against CD40L and CD40^{-/-} mice were immunized with OVA and ISS-ODN as described in Fig. 1 legend. CD40^{-/-} (◆) mice and α-CD40L-treated mice (●) primed similar levels of CTL activity compared to wt mice (■), indicating that CD40/ CD40L does not play a significant role in the activation step of cross-priming. (B) B7/ CD28 is the dominant costimulus in the activation step of cross-priming. WT mice pretreated with mAb blockade against B7-1 and -2 +/- mAb blockade against CD40L and CD28^{-/-} mice +/- mAb blockade against CD40L were immunized with OVA and ISS-ODN as described in Fig. 1 legend. Blockade of B7 signaling (●) resulted in 52% reduction in CTL priming at 25:1 effector:target (E:T) ratio and 80% reduction in priming at 1:1 E:T ratio compared to control animals (■). Immunization of CD28^{-/-} mice (▲) resulted in similar reductions. Additional blockade of CD40L did not alter these responses, indicating that co-stimulation at the activation step is mediated primarily by B7-1 and -2/ CD28 signaling, and that CD40/ CD40L does not make a significant contribution. Adapted with permission from ref. (32).

priming of CTL in this system, it does not synergize with the dominant B7 costimulus. Vaccination of mice lacking ICAM-1, an adhesion molecules that is also upregulated on APC by ISS stimulation, with inactivated tumor cells results in no antigen-specific CTL priming (39). However, this effect can be overcome with the addition of CD40 co-stimulation, supporting the model that CD40-dependent upregulation of B7 molecules in the licensing

step is the dominant co-stimulus at activation. Ex vivo analysis suggests that ICAM-1/ LFA-1 interaction contributes to cross-priming by facilitating accumulation of CTL in proximity to APC.

3. DISCUSSION

ISS-based vaccines facilitate complementary functions of cross-presentation and costimulation, providing a “licensed” APC ready to prime CTL. Cross-presentation appears to be achieved by TAP-dependent endosome-to-cytosol shuttling, resulting in MHC class I-restricted antigen presentation. Costimulation is mediated primarily via B7/CD28 interactions, with non-synergistic contributions by IL-12 and ICAM-1. These data illuminate the role of ISS in priming antigen-specific CTL activity and its relationship to in vivo cross-priming.

Because they are independent of T-cell help, experiments with ISS-based vaccines allow further refinement of the licensing model of cross-priming because they isolate the interaction between licensed APC and CTL. ISS delivered with exogenous antigen act upon APC by facilitating cross-presentation, possibly via increased expression of TAP and MHC class I, and concomitant upregulation of B7 molecules to provide the dominant co-stimulatory signal and IL-12 and ICAM-1 for accessory costimulation (Fig.5, path 1). These functions replace the CD40/CD40L-dependent licensing interaction between T helper cells and APC (Fig. 5, path 2). These data suggest the following sequence for in vivo cross-priming. Pathogen-associated antigens, such as material from lysed, virus-infected cells, are phagocytized by APC for presentation on MHC class II. Antigen-specific T helper cells recognize class II-restricted antigen and license the APC via CD40/ CD40L, which results in upregulation of costimulatory molecules and possibly facilitation of cross-presentation on MHC class I. This in turn leads to priming of naive CD8⁺ T cells, resulting in expansion of an antigen-specific CTL response.

This model provides a mechanistic explanation for the greater efficiency of protein and ISS-ODN vaccines in cross-priming (12–14). Protein and ISS-ODN vaccines are more efficient than plasmid DNA vaccines because they reliably deliver high local concentrations of antigen to the APC. In contrast, production of antigen from the cDNA template in plasmid DNA is variable. ISS-based vaccines are more efficient than protein antigen with or without haptens and adjuvants because they provide the licensing stimulus to APC that favors CTL priming and a T_h1-type immune response.

This model highlights important questions regarding ISS in the priming of CTL. First, how does ISS stimulation increase the expression of TAP? Both IFN α and TNF α can influence TAP expression in APC (40,41).

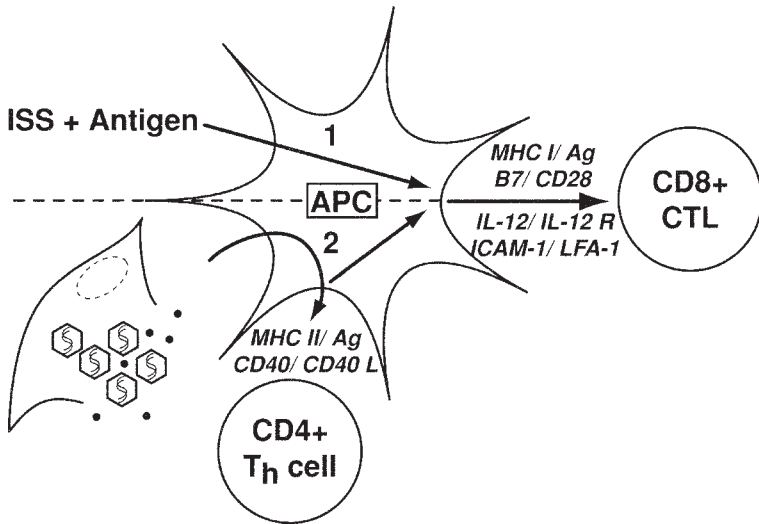


Fig. 5. Complementary functions of ISS in cross-priming CTL. ISS delivered with antigen efficiently prime CTL activity by inducing complementary functions of TAP-dependent cross-presentation and B7, IL-12, and ICAM-1 costimulation (path 1). Antigenic peptides are cross-presented on MHC class I for recognition by TCR on naive CTL, and the costimulatory molecules, primarily B7-1 and -2, provide the necessary second signal for cross-priming. In this model, ISS replaces the CD40/ CD40L-dependent licensing interaction between T_h cells and APC (path 2).

Autocrine signaling via these cytokines also participates in the maturation of DC precursors (42). Is it therefore possible that ISS regulates TAP expression via autocrine signaling via IFN α and/ or TNF α ? Second, what are the intracellular signaling pathways that mediate the effects of ISS on cross-presentation and costimulation? Two distinct signaling molecules, DNA-dependent protein kinase (DNA-PK) and Tlr9, a member of the Toll-like receptor family, appear to have primary roles in the activation of innate immunity by ISS DNA (43,44). In addition, receptor-associated adapter proteins such as TNF receptor associated factor 6 (TRAF6) and myeloid differentiation marker 88 (MyD88) appear to participate in ISS signaling (45). The association of these disparate elements in the action of ISS is as yet undefined. Ongoing investigations are attempting to define the molecular mechanisms that translate recognition of ISS sequences into a favorable milieu for cross-priming antigen specific CTL.

ISS-based vaccines have been invaluable as a molecular probe into the mechanisms of cell-mediated immunity. In addition, these vaccines have demonstrated activity in stimulating protective, antigen-specific CTL immunity in animal models of cancer (13). Because of their unique ability to coordinate the complementary functions of cross-presentation and costimulation, ISS-based immunization strategies may have future clinical application in the treatment of cancer and other diseases.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants AI40682 and AI47078, and by a grant from Dynavax Technologies Corporation.

REFERENCES

1. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.
2. Krieg, A. M., Yi, A. K., Matson, S., (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
3. Ballas, Z.K., Rasmussen, W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
4. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M.-D., Kornbluth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
5. Raz, E., Tighe, H., Sato, Y., et al. (1996) Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Nat. Acad. Sci. USA* **93**, 5141–5145.
6. Roman, M., Martin-Orozco, E., Goodman, J. S., (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
7. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V., and Harding, C. V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 immunity. *J. Exp. Med.* **186**, 1623–1631.
8. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3046.
9. Corr, M., Lee, D. J., Carson, D. A., and Tighe, H. (1996) Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* **184**, 1555–1560.
10. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T

- cell responses to protein antigen: a new class of vaccine adjuvants. *Europ. J. Immunol.* **27**, 2340–2344.
11. Weiner, G. J., Liu, H.-M., Wooldridge, J. E., Dahle, C. E. and Krieg, A. M. (1997) Immunostimulatory oligodeoxynucleotides containing CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Nat. Acad. Sci. USA* **94**, 10,833–10,837.
 12. Horner, A. A., Ronaghy, A., Cheng, P. M., (1998) Immunostimulatory DNA is a potent mucosal adjuvant. *Cell. Immunol.* **190**, 77–82.
 13. Cho, H. J., Takabayashi, K., Cheng, P.-M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotech.* **18**, 509–514.
 14. Tighe, H., Takabayashi, K., Schwartz, D., (2000) Conjugation of protein to immunostimulatory DNA results in rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **7**, 1939–1947.
 15. Davis, H. L., Weeratna, R., Waldschmidt, T. J., (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
 16. Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M.-D., Silverman, G. J., Lotz, M., Carson, D. A., and Raz, E. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
 17. Keene, J.-A., and Forman, J. (1982) Helper activity is required for the in vivo generation of cytotoxic lymphocytes. *J. Exp. Med.* **155**, 768–782.
 18. Sperling, A. I., and Bluestone, J. A. (1996) The complexities of T-cell co-stimulation: CD28 and beyond. *Immunol. Rev.* **153**, 155–182.
 19. Chambers, C. A., and Allison, J. P. 1997. Co-stimulation in T cell responses. *Curr. Opin. Immunol.* **9**, 396–404.
 20. Lanzavecchia, A. (1998) License to kill. *Nature* **393**, 413–414.
 21. Ridge, J. P., Di Rosa, F., and Matzinger, P. 1998. A conditioned dendritic cell can be a bridge between CD4⁺ T-helper and a T-killer cell. *Nature* **393**, 474–478.
 22. Schoenberger, S. P., Toes, R. E. M., van der Voort, E. I. H., Offringa, R. and Melief, C. J. M. (1998) T-cell help for cytotoxic lymphocytes is mediated by CD40-CD40L interactions. *Nature* **393**, 480–483.
 23. Bennett, S. R., Carbone, F. R., Karamalis, F., Flavell, R. A., Miller, J. F., and W. R. Heath. (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signaling. *Nature* **393**, 478–480.
 24. Kovacsovics-Bankowski, M. and Rock, K. L. (1995) A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**, 243–246.
 25. Shen, Z., Reznikoff, G., Dranoff, G., and Rock, K. (1997) Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J. Immunol.* **158**, 2723–2730.
 26. Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J., and Harding, C. V. (1993) Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361**, 359–362.

27. Reis e Souza, C., and Germain, R. N. (1995) Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J. Exp. Med.* **182**, 841–851.
28. Gromme, M., Uytdehaag, F. G., Janssen, H. (1999) Recycling MHC class I molecules and endosomal peptide loading. *Proc. Nat. Acad. Sci. USA* **96**, 10,326–10,331.
29. Sigal, L. J., Crotty, S., Andino, R., and Rock, K. L. (1999) Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* **398**, 77–80.
30. Sigal, L. J., and Rock, K. L. (2000) Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. *J. Exp. Med.* **192**, 1143–1159.
31. Sandberg, J. K., Chambers, B. J., Van Kaer, L., Kearre, K., and Ljunggren, H. G. (1996) TAP1-deficient mice select a CD8+ T cell repertoire that displays both diversity and peptide specificity. *Eur. J. Immunol.* **26**, 288–293.
32. Cho, H. J., Hayashi, T., Datta, S. K., Takabayashi, K., Van uden, J. H., Horner, A., Corr, M., and Raz, E. (2002). IFN- $\alpha\beta$ promote priming of antigen-specific CD8(+) and CD4(+) T lymphocytes by immunostimulatory DNA-based vaccines. *J. Immunol.* **168**, 4907.
33. Grewal, I. S., and Flavell, R. A. (1996) The role of CD40 ligand in costimulation and T-cell activation. *Immunol. Rev.* **153**, 85–106.
34. Harding, F. A., and Allison, J. P. (1993) CD28-B7 interactions allow the induction of CD8+ cytotoxic T lymphocytes in the absence of exogenous help. *J. Exp. Med.* **177**, 1791–1796.
35. Sigal, L. J., Reiser, H., and Rock, K. L. (1998) The role of B7-1 and B7-2 costimulation for the generation of CTL responses in vivo. *J. Immunol.* **161**, 2740–2745.
36. Trinchieri, G. (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Ann. Rev. Immunol.* **13**, 251–276.
37. Linsley, P.S., Greene, J. L., Brady, W., Bajorath, J., Ledbetter, J. A., and Peach, R. (1994) Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* **1**, 793–801.
38. Yang, Y. and Wilson, J. M. (1996) CD40 Ligand-Dependent T Cell Activation: Requirement of B7-CD28 Signaling Through CD40. *Science* **273**, 1862–1864.
39. Schoenberger, S. P., van Stipdonk, M. J. B., Prilliman, K. R., and Lemmens, E. (2000) The role of B7, ICAM-1, and APC activation in cross-priming of cytotoxic T lymphocytes. *Cancer Vaccines 2000*, New York, NY.
40. Brossart, P. and Bevan, M. (1997) Presentation of exogenous protein antigens on major histocompatibility complex class I molecules by dendritic cells: pathway of presentation and regulation by cytokines. *Blood* **90**, 1594–1599.
41. Ma, W., Lehner, P. J., Cresswell, P., Pober, J. S., and Johnson, D. R. 1997. Interferon-gamma rapidly increases peptide transporter (TAP) subunit expression and peptide transport capacity in endothelial cells. *J. Biol. Chem.* **272**, 16,585–16,590.

42. Kadowaki, N., Antonenko, S., Lau, J. Y.-N., and Liu, Y.-J. 2000. Natural interferon α/β -producing cells link innate and adaptive immunity. *J. Exp. Med.* **192**, 219–225.
43. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
44. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
45. Hacker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., and Wagner, H. (2000) Immune cell activation by bacterial CpG-DNA through myeloid differentiation marker 88 and tumor necrosis factor receptor-associated factor (TRAF) 6. *J. Exp. Med.* **192**, 595–600.

PART IV

VACCINATION STRATEGIES

The Th1 Adjuvant Effect of Immunostimulatory (ISS) DNA Sequences

Maripat Corr and Chih Min Tang

1. INTRODUCTION

In the seminal study by Wolff et al., plasmid or naked DNA immunization injected directly into the muscle of mice could induce expression of the reporter gene encoded by the plasmid injected into the muscle cell (1). Subsequent studies revealed that this form of gene transfer could result in a protective immune response to influenza (2,3). The basis of this response was in the generation of antigen specific cytotoxic T lymphocyte (CTL) and humoral responses (2–4). Further studies demonstrated that these adaptive immune responses were associated with an antibody isotype and cytokine profile consistent with a strong Th1 response (5,6).

Activated CD4+ T helper cells can largely be divided into two subsets. The T helper 1 (Th1) subset produce lymphotoxin β , IFN γ , and IL-2 whereas the Th2 subset expresses IL-4, IL-5, and IL-13 (7). Although several factors have been shown to influence the phenotype of the T helper cell response, the initial cytokine milieu at the time of T-cell priming appears to be of central importance (8). IL-12 is key in the development of a Th1 response, and IL-4 facilitates Th2 differentiation (7). DNA vaccines largely exert their adjuvant activity through their interaction with cells of the innate immune response elements causing these cells to release tumor necrosis factor (TNF) α , IFN γ and IL-12 (9). These cytokines are then felt to be instrumental in establishing a Th1 response. However, coimmunization strategies with Th2 cytokine expressing plasmids can overcome this propensity for generating Th1 dominated responses.

2. TH1 EFFECTS OF DNA IMMUNIZATION

The immunogenicity of plasmid DNA was localized to the backbone of the plasmid vector (10). When kanamycin resistance genes were exchanged for the ampicillin cassette, the immune response to the encoded antigen was diminished (10). The difference was attributed to lie in the number of hexamer motifs containing a central CpG in the nonexpressing portion of the vector. CpG dinucleotides are abundant in bacterial and invertebrate genomes, but are present at lower than expected frequency in vertebrate genomes (11). In mammals, these cytosine containing sites are largely methylated as epigenetic mechanisms of transcriptional regulation, X chromosome inactivation or suppression of endogenous viruses (12–14). Unmethylated CpG dinucleotides nested within particular flanking sequences are recognized as foreign and activate first line defense elements or the innate immune system. These motifs were found to have potent adjuvant effects by virtue of the responses they triggered in the innate immune arm.

Although these CpG containing motifs or ISS were originally identified in mycobacterial DNA, similar responses could be elicited by using these sequences in synthetic nuclease resistant phosphorothioate based oligonucleotides (ODN) (15–17). The presence of ISS in plasmids was recognized as a potent adjuvant in stimulating the immune response (10). Extending these observations, CpG containing ODN administered with antigen or chemically linked to antigen was more potent than plasmid inoculation in raising Th1 cellular and associated antibody responses (18,19). It stands to reason that the innate defense that has evolved to recognize CpG and protect against invading pathogens would trigger a Th1 response (9,20–24).

An innate immune receptor for CpG DNA has been identified in the Toll-like receptor family, TLR9 (25). TLR9-deficient mice do not respond to ISS-ODN, including proliferation of splenocytes, inflammatory cytokine production from macrophages, and maturation of dendritic cells. The *in vivo* CpG-DNA-mediated Th1 response was also abrogated in TLR9-deficient mice (25). Other signaling pathways have been associated with the inflammatory responses elicited by ISS. CpG-DNA induces phosphorylation of Jun N-terminal kinase kinase 1 (JNKK1/SEK/MKK4) and subsequent activation of the stress kinases JNK1, JNK2, and p38 in murine macrophages and dendritic cells (26,27). This leads to phosphorylation of c-Jun and ATF2, activating transcription factor activating protein-1 (AP-1) (26). The stress kinase activation cascade is essential for CpG-DNA-induced cytokine release of TNF α and IL-12 (26).

In addition, ISS-ODN and bacterial DNA activate DNA-PK, which in turn contributes to activation of IKK and NF-kappaB (28–30). DNA-PK is a protein kinase that plays a pivotal role in the repair of DNA double-stranded breaks from intrinsic cellular processes or environmental insults (31,32). In vitro stimulation of bone marrow derived macrophages from mice lacking the catalytic subunit of DNA-PK (DNA-PKcs) with ISS-ODN results in defective induction of IL-6 and IL-12 (29). These results implicate a role for DNA-PKcs in innate immune responses and a link between DNA repair and innate immunity (29).

In the bridge from innate immunity to adaptive immunity T cells, however, are not directly stimulated by ISS (33). The differentiation of naive cells is largely influenced by the cytokine milieu of the priming environment (7). The effect of ISS stimulation on T-cell antigen recognition appears to be indirect and at least in part mediated by type I IFN (34). ISS-ODNs stimulate responses to antigen by initiating IFN γ , IFN α , IFN β , IL-12, and IL-18 production fostering Th1 responses and the enhancement of cell mediated immunity (9). The production of IL-12 in particular would strongly bias the CD4+ T cell phenotype to a Th1 or IFN γ secreting response (7).

The effect of ISS exposure is predominantly mediated by the response of antigen presenting cells to CpG-DNA. ISS treatment is a particularly powerful inducer of IL-12 from macrophages and dendritic cells (21,22,35–38). ISS activated monocytes and macrophages also produce the type I cytokines IL-12, IL-18, IFN α/β as well as TNF α (9). Other cytokines that are induced include IL-6, IL-1 β , IL-1RA, MIP-1 β , and MCP-1 (9,15,34,39–42). This response profile is not limited to murine cells as the induction of TNF α , IL-6, and IFN γ secretion has also been detected using human monocytes and peripheral blood mononuclear cells (43,44). The antigen presenting function of these cells is further potentiated by the enhanced expression of surface molecules associated with priming an immune response (45). The functional capacity of these antigen presenting cells (APCs) is then enhanced as reflected by their improved ability to stimulate alloreactive T cells (37).

Other elements of the innate immune system further augment the effect of ISS stimulation. Purified natural killer (NK) cells are not directly activated by ISS, but the IL-12, TNF α , and IFN α/β produced by monocytes or macrophages or other APCs do result in their activation (46). NK cells then secrete a large amount of IFN γ into the local environment that would perpetuate a Th1 response. In kind, the ISS activation of macrophages is quantitatively enhanced by the IFN γ produced by NK cells in a feedback loop (16). Thus, ISS is able to activate the innate immune response in such a way, as to skew the adaptive response toward Th1 in a manner that provides self-amplifying cytokine cascades.

3. TH2 EFFECTS OF DNA IMMUNIZATION

DNA vaccination under certain circumstances can induce a qualitative Th2 response. The phenotype of the immune response can be manipulated by the route of administration as well as by adding a cytokine expressing construct as a coinoculation. Administration of plasmid DNA using a gene gun can result in Th2 responses (47). Gene gun technology uses a gas driven biolistic bombardment that propels gold particles coated with plasmid DNA into the cytosol of cells in the skin. Repetitive administration of plasmid DNA with a gene gun has been shown to increase IL-4 production while concomitantly decreasing IFN γ production (47). The antibody profile using this method was concordantly IgG1 specific, whereas intramuscular injection resulted in a predominantly IgG2a isotype response. The Th2 profile of the response was not limited to cutaneous gene gun administration, but also occurred if surgically exposed muscle was inoculated (48).

Biolistic administration of DNA vaccines is a powerful influence on the induction of the Th2 response. The ability of gene gun to "circumvent" the Th1 induction by CpG containing DNA may lie in the basic mechanics of this technology. The gold particles and consequently the DNA are directly delivered into the cytosol of the cell. The DNA is protected from exposure to the innate arms of the immune system to secrete TNF α , IL-12, or IFN γ . Another potential mechanism for this difference is the availability of antigen to different dendritic cells. Subclasses of dendritic cells have been described that differentially regulate the type of Th response (49–52). Biolistic administration may deliver the plasmid directly to the dendritic cell subset which initiates Th2 predominant responses. The form of antigen may also select the pattern of cytokine secretion by the dendritic cell, which would also bias the priming environment (53).

A more direct approach to biasing the immune response to DNA vaccination has been to coadminister plasmids that express known Th2 biasing cytokines like IL-4. Using IL-4 as a cytokine adjuvant results in enhanced proliferation, the expression of Th2 cytokines by T cells, and the production of an IgG1 isotype predominant antibody response (54–59). Surprisingly, the coadministration of a granulocyte-macrophage colony-stimulating factor (GM-CSF) expressing plasmid also has been reported to give a Th2 response (60). This response, however, is mixed in some studies (54,57–59,61–63).

4. THE TH1 EFFECT IS PROTECTIVE IN ANIMAL ALLERGY MODELS

The Th1 bias has applications not only in vaccination, but also in therapy for allergy, which is a Th2 dominated process. Immunization with plasmid

DNA was first used to show a reversal of an antigen specific Th2 response to a Th1 response (64). Coadministration of ISS ODN can redirect a Th2 response to hen egg lysozyme, and IFA toward a Th1 response, as evidenced by cytokine release and antibody isotype (20). In a murine asthma model systemic administration of ISS appears beneficial in animals previously sensitized with schistosoma eggs that normally result in airway hypereosinophilia, IgE production, and bronchial hyper-reactivity (65). This effect appears however to be IL-12 and IFN γ independent (66). In a different murine model immediate and sustained protection against allergen induced airway hypersensitivity could be conferred by ISS administration (67). Although the immediate effects were thought to be owing to IL-12 and IFN release, the sustained effect was felt to be through reduced peripheral eosinophils (67). These studies demonstrated the potential benefits of utilizing the Th1 promoting effect of ISS to alter the Th1/Th2 balance to reverse Th2 biased disease states.

5. CONCLUDING COMMENTS

ISS owing to a wide range of stimulatory effects, has been found to be a potent adjuvant in inducing Th1 immune responses. Direct stimulation of macrophages and dendritic cells causes the release of IL-12 and other Th1 promoting factors. This further stimulates NK cells to participate through the release of IFN γ . This effect bridges the innate and adaptive immune arms in host defenses through establishing a cytokine environment that promotes Th1 responses. The use of ISS as adjuvants has therapeutic implications for the development of vaccines to combat infections or neoplasms. Alternatively, ISS may become an effective immunomodulator of disease states that are predominantly Th2 mediated atopic diatheses.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (AI40682, AR40770, AR44850). We are grateful to N. Noon and J. Uhle for secretarial assistance.

REFERENCES

1. Wolff, J. A., Malone, R. W., Williams, P., et al. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465–1468.
2. Robinson, H. L., Hunt, L. A., and Webster, R. G. (1993) Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine* **11**, 957–960.
3. Ulmer, J. B., Donnelly, J. J., Parker, S. E., et al. (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745–1749.

4. Justewicz, D. M., Morin, M. J., Robinson, H. L., and Webster, R. G. (1995) Antibody-forming cell response to virus challenge in mice immunized with DNA encoding the influenza virus hemagglutinin. *J. Virol.* **69**, 7712–7717.
5. Deck, R. R., DeWitt, C. M., Donnelly, J. J., Liu, M. A., and Ulmer, J. B. (1997) Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* **15**, 71–78.
6. Raz, E., Carson, D. A., Parker, S. E., et al. (1994) Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* **91**, 9519–9523.
7. Seder, R. A. and Paul, W. E. (1994) Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu. Rev. Immunol.* **12**, 635–673.
8. O'Garra, A. (1998) Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**, 275–283.
9. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
10. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
11. Bird, A. P. (1980) DNA methylation and the frequency of CpG in animal DNA. *Nucleic Acids Res.* **8**, 1499–1504.
12. Bird, A. P. (1993) Functions for DNA methylation in vertebrates. *Cold Spring Harb. Symp. Quant Biol.* **58**, 281–285.
13. Ng, H. H. and Bird, A. (1999) DNA methylation and chromatin modification. *Curr. Opin. Genet Dev.* **9**, 158–163.
14. Tate, P. H. and Bird, A. P. (1993) Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Genet Dev.* **3**, 226–231.
15. Yamamoto, S., Kuramoto, E., Shimada, S., and Tokunaga, T. (1988) In vitro augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **79**, 866–873.
16. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.
17. Zhao, Q., Matson, S., Herrera, C. J., Fisher, E., Yu, H., and Krieg, A. M. (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res. Dev.* **3**, 53–66.
18. Cho, H. J., Takabayashi, K., Cheng, P. M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514.
19. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947.

20. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V., and Harding, C. V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* **186**, 1623–1631.
21. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344.
22. Lipford, G. B., Sparwasser, T., Bauer, M., Zimmermann, S., Koch, E. S., Heeg, K., and Wagner, H. (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* **27**, 3420–3426.
23. Weiner, G. J., Liu, H. M., Wooldridge, J. E., Dahle, C. E., and Krieg, A. M. (1997) Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. USA* **94**, 10,833–10,837.
24. Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J., and Krieg, A. M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
25. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
26. Hacker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.
27. Yi, A. K. and Krieg, A. M. (1998) Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493–4497.
28. Yi, A. K. and Krieg, A. M. (1998) CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. *J. Immunol.* **160**, 1240–1245.
29. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
30. Stacey, K. J., Sweet, M. J., and Hume, D. A. (1996) Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116–2122.
31. Carter, T., Vancurova, I., Sun, I., Lou, W., and DeLeon, S. (1990) A DNA-activated protein kinase from HeLa cell nuclei. *Mol. Cell Biol.* **10**, 6460–6471.
32. Smith, G. C. and Jackson, S. P. (1999) The DNA-dependent protein kinase. *Genes Dev.* **13**, 916–934.
33. Bendigs, S., Salzer, U., Lipford, G. B., Wagner, H., and Heeg, K. (1999) CpG-oligodeoxynucleotides co-stimulate primary T cells in the absence of antigen-presenting cells. *Eur. J. Immunol.* **29**, 1209–1218.
34. Sun, S., Zhang, X., Tough, D. F., and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* **188**, 2335–2342.
35. Chace, J. H., Hooker, N. A., Mildenstein, K. L., Krieg, A. M., and Cowdery, J. S. (1997) Bacterial DNA-induced NK cell IFN-gamma production is dependent on macrophage secretion of IL-12. *Clin. Immunol. Immunopathol.* **84**, 185–193.

36. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
37. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C., and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3049.
38. Jakob, T., Walker, P. S., Krieg, A. M., von Stebut, E., Udey, M. C., and Vogel, J. C. (1999) Bacterial DNA and CpG-containing oligodeoxynucleotides activate cutaneous dendritic cells and induce IL-12 production: implications for the augmentation of Th1 responses. *Int. Arch. Allergy Immunol.* **118**, 457–461.
39. Yi, A. K., Chace, J. H., Cowdery, J. S., and Krieg, A. M. (1996) IFN- γ promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J. Immunol.* **156**, 558–564.
40. Yi, A. K., Klinman, D. M., Martin, T. L., Matson, S., and Krieg, A. M. (1996) Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* **157**, 5394–5402.
41. Schwartz, D. A., Quinn, T. J., Thorne, P. S., Sayeed, S., Yi, A. K., and Krieg, A. M. (1997) CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* **100**, 68–73.
42. Zhao, Q., Temsamani, J., Zhou, R. Z., and Agrawal, S. (1997) Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. *Antisense Nucleic Acid Drug Dev.* **7**, 495–502.
43. Hartmann, G. and Krieg, A. M. (1999) CpG DNA and LPS induce distinct patterns of activation in human monocytes. *Gene Ther.* **6**, 893–903.
44. Yamamoto, T., Yamamoto, S., Kataoka, T., Komuro, K., Kohase, M., and Tokunaga, T. (1994) Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. *Jpn. J. Cancer Res.* **85**, 775–779.
45. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M. D., Kornbluth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
46. Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
47. Pertmer, T. M., Roberts, T. R., and Haynes, J. R. (1996) Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J. Virol.* **70**, 6119–6125.
48. Feltquate, D. M., Heaney, S., Webster, R. G., and Robinson, H. L. (1997) Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* **158**, 2278–2284.

49. Pulendran, B., Smith, J. L., Caspary, G., et al. (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc. Natl. Acad. Sci. USA* **96**, 1036–1041.
50. Rissoan, M. C., Soumelis, V., Kadowaki, N., et al. (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283**, 1183–1186.
51. Maldonado-Lopez, R., De Smedt, T., Michel, P., et al. (1999) CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med* **189**, 587–592.
52. Smith, A. L., and de St Groth, B. F. (1999) Antigen-pulsed CD8alpha+ dendritic cells generate an immune response after subcutaneous injection without homing to the draining lymph node. *J. Exp. Med.* **189**, 593–598.
53. d'Ostiani, C. F., Del Sero, G., Bacci, A., et al. (2000) Dendritic cells discriminate between yeasts and hyphae of the fungus *Candida albicans*. Implications for initiation of T helper cell immunity in vitro and in vivo. *J. Exp. Med.* **191**, 1661–1674.
54. Maecker, H. T., Umetsu, D. T., DeKruyff, R. H., and Levy, S. (1997) DNA vaccination with cytokine fusion constructs biases the immune response to ovalbumin. *Vaccine* **15**, 1687–1696.
55. Kim, J. J., Trivedi, N. N., Nottingham, L. K., et al. (1998) Modulation of amplitude and direction of in vivo immune responses by co-administration of cytokine gene expression cassettes with DNA immunogens. *Eur. J. Immunol.* **28**, 1089–1103.
56. Raz, E., Watanabe, A., Baird, S. M., et al. (1993) Systemic immunological effects of cytokine genes injected into skeletal muscle. *Proc. Natl. Acad. Sci. USA* **90**, 4523–4527.
57. Geissler, M., Gesien, A., Tokushige, K., and Wands, J. R. (1997) Enhancement of cellular and humoral immune responses to hepatitis C virus core protein using DNA-based vaccines augmented with cytokine-expressing plasmids. *J. Immunol.* **158**, 1231–1237.
58. Kim, J. J., Simbiri, K. A., Sin, J. I., et al. (1999) Cytokine molecular adjuvants modulate immune responses induced by DNA vaccine constructs for HIV-1 and SIV. *J. Interferon Cytokine Res.* **19**, 77–84.
59. Chow, Y. H., Chiang, B. L., Lee, Y. L., et al. (1998) Development of Th1 and Th2 populations and the nature of immune responses to hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine genes. *J. Immunol.* **160**, 1320–1329.
60. Gurunathan, S., Irvine, K. R., Wu, C. Y., et al. (1998) CD40 ligand/trimer DNA enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J. Immunol.* **161**, 4563–4571.
61. Xiang, Z. and Ertl, H. C. (1995) Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* **2**, 129–135.
62. Lewis, P. J., Cox, G. J., van Drunen Littel-van den Hurk, S., and Babiuk, L. A. (1997) Polynucleotide vaccines in animals: enhancing and modulating responses. *Vaccine* **15**, 861–864.

63. Weiss, W. R., Ishii, K. J., Hedstrom, R. C., Sedegah, M., Ichino, M., Barnhart, K., Klinman, D. M., and Hoffman, S. L. (1998) A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. *J. Immunol.* **161**, 2325–2332.
64. Raz, E., Tighe, H., Sato, Y., et al. (1996) Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* **93**, 5141–5145.
65. Kline, J. N., Waldschmidt, T. J., Businga, T. R., et al. (1998) Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555–2559.
66. Kline, J. N. (2000) Effects of CpG DNA on Th1/Th2 balance in asthma. *Curr. Top Microbiol. Immunol.* **247**, 211–225.
67. Broide, D., Schwarze, J., Tighe, H., et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054–7062.

Immunostimulatory DNA Prepriming for the Induction of Th1 and Prevention of Th2 Biased Immune Responses

Hiroko Kobayashi, Elena Martin-Orozco,
Kenji Takabayashi and Anthony A. Horner

1. INTRODUCTION

Early reports on the immunologic activity of ISS-ODN focused on its ability to induce natural killer (NK) cell activation, B-cell proliferation, and the production of type-1 cytokines such as IL-12, type 1 IFNs, and IFN- γ from antigen-presenting cells (APCs), B cells, and NK cells in an antigen independent manner (1–4). In addition to these effects, ISS-ODN has more recently been shown to increase the expression of various costimulatory molecules on APCs and B cells (Figs. 1 A,B) (5–7). Interestingly, although ISS-ODN induces an innate cytokine response and increased costimulatory molecule expression by several cell types, ISS-ODN does not appear to activate T cells directly (6,8).

Our experience is that when splenocytes are incubated with ISS-ODN, cytokine levels in supernatants and cell surface expression of costimulatory molecules are maximal after 72 and 48 h, respectively (data not shown). These results suggested that ISS-ODN might have a prolonged effect on the innate immune system. To better understand the time course of ISS-ODN activation of the innate immune system, we conducted *in vivo* experiments. Our results demonstrated that ISS-ODN induced increased cytokine production in injected mice for approx two wk (Table 1) (8). Increased expression of costimulatory molecules on the surface of immunocytes of ISS-ODN injected mice lasted for about the same period of time (Fig. 2) (6).

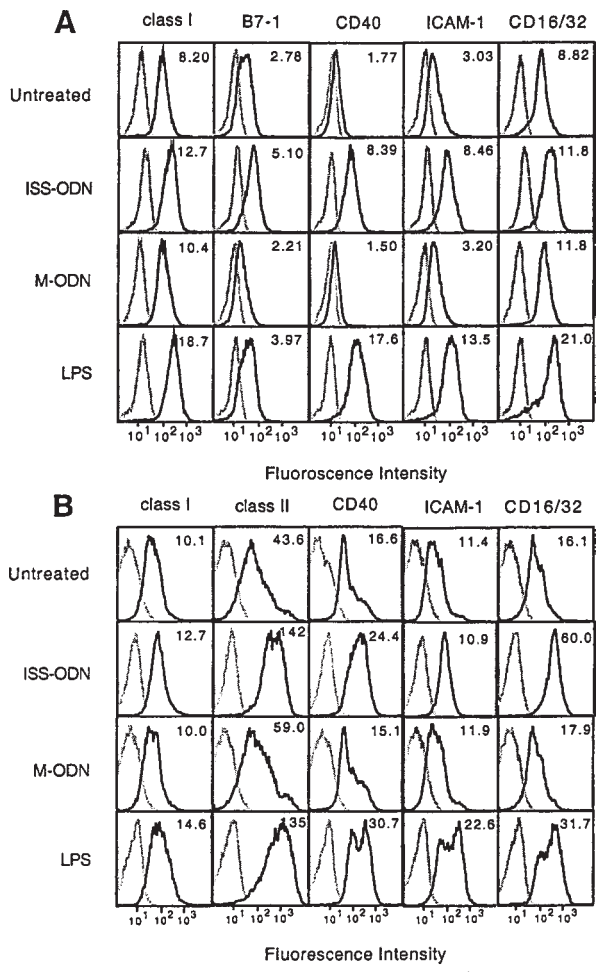


Fig. 1. ISS-ODN effect on bone marrow derived macrophage and B-cell expression of costimulatory molecules in vitro. Purified bone marrow derived macrophages (BMDM; $2 \times 10^5/\text{mL}$) or splenic B cells ($2 \times 10^6/\text{mL}$) were incubated with ISS-ODN: 5'-TGACTGTGAACGTTTCG-AGATGA-3' ($1 \mu\text{g}/\text{mL}$), mutated (M)-ODN: 5'-TGACTGTGAACCTTAGAGATGA-3' ($1\mu\text{g}/\text{mL}$), LPS ($5\mu\text{g}/\text{mL}$), or were left unstimulated. After 48 h cells were stained and subjected to FACs analysis. On the x-axis a log scale of fluorescence intensity for each surface molecule is presented, and the Y-axis represents the number of Mac3⁺ (BMDM) or B220⁺ (B cells) cells found at that fluorescence intensity. For each histogram, the surface molecule specific antibody (black line) is compared to the appropriate isotype control (gray line). The number in the right upper corner of each graph is the mean fluorescence intensity ratio (MFIR). MFIR = (surface molecule specific mean fluorescence intensity)/(isotype control mean fluorescence intensity) (6). (A). BMDM, (B). Splenic B cells.

Table 1
In vivo Cytokine Production Induced by ISS-ODN

ISS-ODN	IL-12 (pg/ml)	IFN- γ (pg/ml)
	<42	<14
Day (-1)	4028 $\pm$ 1878	343 $\pm$ 83
Day (-3)	2356 $\pm$ 464	312 $\pm$ 101
Day (-7)	2034 $\pm$ 288	174 $\pm$ 36
Day (-14)	763 $\pm$ 255	35 $\pm$ 71
LPS Day (-7)	<42	<14

At serial time, points after mice were injected id with ISS-ODN (50 μ g) or LPS (10 μ g), serum was obtained and cytokine levels were analyzed by ELISA. Results represent the mean $\pm$ SE for three mice in each time point. Injection of M-ODN did not result in any detectable IL-12 or IFN- γ production (13).

**2. PROLONGED ACTIVATION OF APCs BY ISS-ODN
PLAYS A CENTRAL ROLE IN ITS MEDIATION OF TH1
BIASED ADAPTIVE IMMUNE RESPONSES**

The functionality of APC activation by ISS-ODN on T-cell responses has been confirmed in experiments utilizing TCR-OVA transgenic (TG) mice (DO.11.10 mice) (6). Splenic APCs were harvested on d 14 from TCR-OVA TG mice injected with ISS-ODN, mutated oligodeoxynucleotide (M-ODN), or saline on d 0 and 7. Unstimulated T cells were obtained from naive TCR-OVA TG littermates. APCs and T cells were then cultured with ovalbumin (OVA). APCs from ISS-ODN injected mice induced fourfold more T-cell proliferation than splenic APC obtained from mice injected with M-ODN or saline (Fig. 3A). Furthermore, incubation of APCs from ISS-ODN injected mice with naive TCR-OVA TG T cells and OVA resulted in antigen specific IFN- γ production (Fig. 3B), indicating the differentiation of naive Th cells toward a Th1 phenotype (6). Therefore, it appears that ISS-ODN functions as a Th1 adjuvant in large part via the induction of an innate immune response involving APCs. These results further demonstrate that exposure of APCs to ISS-ODN enhances their ability to induce T cell responses for at least 7 d.

**3. ISS-ODN PREPRIMING TH1 BIASES IMMUNE RESPONSES
FOR A PROLONGED PERIOD OF TIME**

Given that ISS-ODN can activate the innate immune system for up to two wk, we hypothesized that injection of mice with ISS-ODN would Th1 bias adaptive immune responses for an extended period of time (ISS-ODN prepriming). The data presented here demonstrate that ISS-ODN provides a

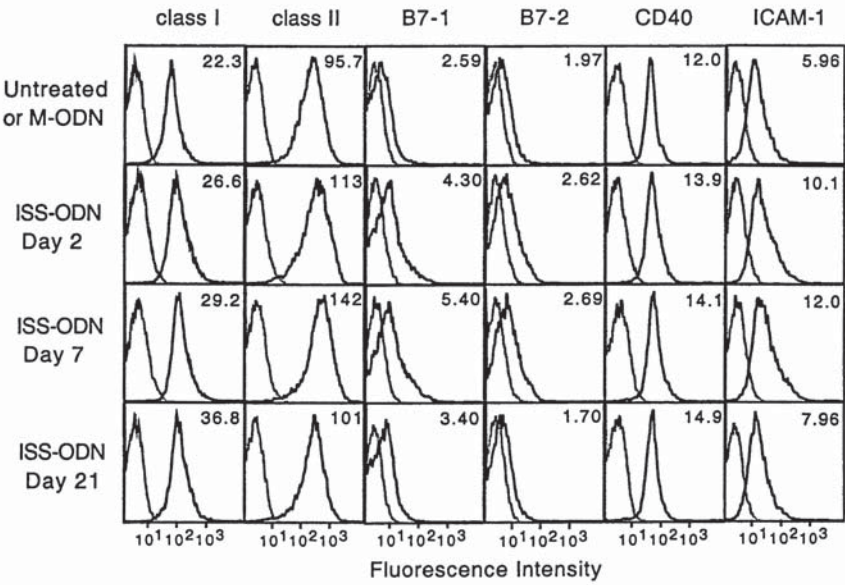


Fig. 2. ISS-ODN effect on in vivo B cell costimulatory molecule expression. Mice were injected ip with ISS-ODN (100 μ g), M-ODN: 5'- (100 μ g) or saline. Mice were sacrificed on day 2, 7, or 21 after injection, splenocytes were harvested and stained for FACs analysis as outlined in the Fig. 1 legend (6).

14-d window of Th1 adjuvant activity for antigens injected into the same site (8). Mice were injected id with ISS-ODN from 14 to 1 d prior to β -galactosidase (β -gal), or with β -gal. When compared to mice id immunized with β -gal alone, mice receiving id ISS-ODN up to 14 d prior to β -gal had a significant increase ($p < 0.05$) in their serum IgG2a response (Table 2). Furthermore, the IgG2a response was improved over ISS-ODN/ β -gal coimmunization if ISS-ODN was given 3–7 d before antigen ($p < 0.05$ for day –7 ISS-ODN delivery vs ISS-ODN/ β -gal coimmunization). In contrast to the relatively IFN- γ dependent IgG2a response, which increased, the relatively IL-4 dependent IgG1 response was not effected by ISS-ODN prepriming or by codelivery with β -gal (data not shown) (8,9).

ISS-ODN has previously been reported to induce a Th1 cytokine profile and a cytotoxic T lymphocyte (CTL) response to soluble protein antigens (4,10–14). Therefore, the ISS-ODN pre-priming effect on cellular immunity was investigated (8). As shown in Table 2, antigen specific IFN- γ production was significantly increased in mice preprimed with ISS-ODN up to 14 d before β -gal immunization compared to mice immunized with antigen alone

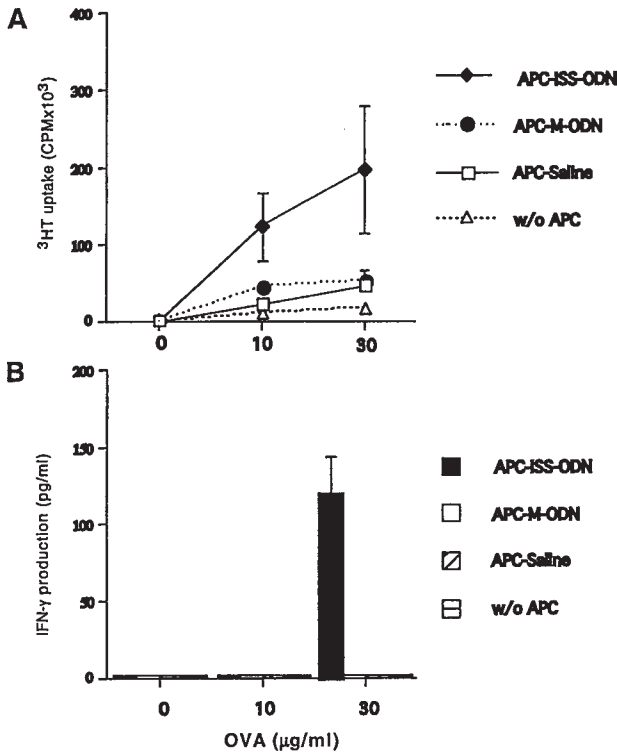


Fig. 3. ISS-ODN effect on APC function. APCs and TCR-OVA TG T cells were harvested from (DO11. 10) mice. APCs were harvested on day 14 after id injections of ISS-ODN (50 μg), M-ODN (50mg), or saline on days 0 and 7. T cells were obtained from naive TCR-OVA TG littermates. APCs and TCR-OVA TG T cells) were cultured in supplemented RPMI at a final concentration of 5×10^6 cells/ml (1:1 ratio) with varying concentrations of OVA, for 4 d. Results represent mean values $\pm$ SD for four mice per group and are representative of three similar and independent experiments (6). **(A)** T cell proliferation. Cells were harvested and [^3H] thymidine incorporation was determined after an 18 h incubation with [^3H] thymidine (1 mCi per well). APC obtained from ISS-ODN injected mice induced fourfold more OVA specific T-cell proliferation than APC obtained from mice injected with saline or M-ODN. **(B)** OVA specific cytokine response. Only APC obtained from TCR-OVA TG mice injected with ISS-ODN induced OVA specific IFN- γ production from naive T cells. Levels of IL-5 and IL-4 were below detectable limits in this system.

($p < 0.05$). Furthermore, mice preprimed with ISS-ODN 3–7 d before β -gal immunization demonstrated a 100% increase in their IFN- γ response compared to mice coimmunized with ISS-ODN and β -gal ($p < 0.05$). However,

Table 2
ISS-ODN Prepriming Effect on Immunization with Protein

ISS-ODN	β -gal	IgG2a (U/mL)	IFN γ (pg/mL)
None	+	80 $\pm$ 21	163 $\pm$ 71
Day (0)	+	420 $\pm$ 80	2620 $\pm$ 874
Day (-3)	+	623 $\pm$ 65	5836 $\pm$ 1569
Day (-7)	+	840 $\pm$ 73	5485 $\pm$ 1286
Day (-14)	+	252 $\pm$ 87	3204 $\pm$ 4672
Day (-28)	+	47 $\pm$ 5	1632 $\pm$ 274

Mice received a single id injection with ISS-ODN (50 μ g) either 3, 7, 14, or 28 d before or with β -gal (50 μ g), via the same route (d 0). Control mice received β -gal without ISS-ODN. At week 8, blood was collected for analysis of antigen specific IgG2a levels, and splenocytes were harvested to determine T-cell cytokine responses. Results represent the mean $\pm$ SE for four mice in each group and are representative of three similar and independent experiments.

ISS-ODN preprimed and coimmunized mice did not develop antigen specific IL-4 or IL-5 responses (data not shown) (8). Further studies evaluated the CTL response of ISS-ODN preprimed mice (8). These results again demonstrated a prolonged window of ISS-ODN adjuvant activity. Administration of ISS-ODN up to 14 d before β -gal delivery led to a significantly improved CTL response over β -gal vaccination without adjuvant ($p < 0.05$; Fig. 4). Although ISS-ODN prepriming 3–7 d prior to antigen did not lead to statistically significant increases in CTL activity when compared to ISS-ODN/ β -gal coimmunization, again, there was a trend toward improved responses if ISS-ODN prepriming occurred on these days. Interestingly, when ISS-ODN was injected one wk prior to or simultaneously with β -gal but at two different sites, the adjuvant and prepriming effects of ISS-ODN were not seen, even when the injected dose of ISS-ODN was increased five-fold (data not shown) (8). This observation demonstrates that the ISS-ODN prepriming effect is local and not systemic.

4. ISS-ODN PREPRIMING INDUCES A TEMPORAL WINDOW OF RESISTANCE TO THE DEVELOPMENT OF TH2 BIASED IMMUNE RESPONSES

We next evaluated the potential of ISS-ODN prepriming to prevent the development of Th2 biased immune deviation. Mice were sensitized to OVA and alum (Th2 adjuvant) by id injection. At various times prior to OVA/alum sensitization, mice received ISS-ODN via the same injection route. These studies demonstrated that ISS-ODN prepriming had a marked effect on IgE production if given 1–7 d prior to OVA/alum sensitization

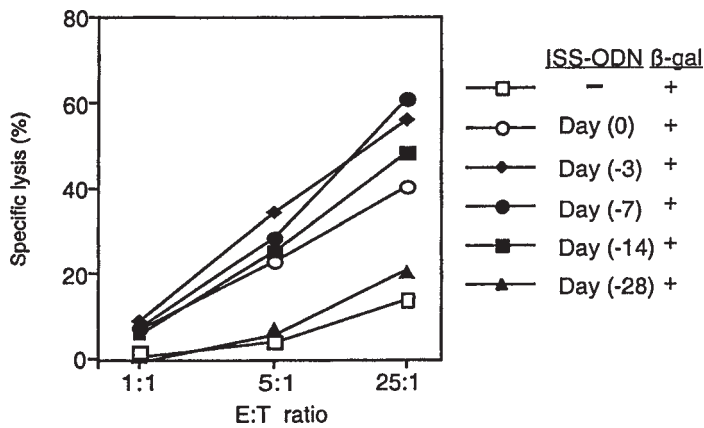


Fig. 4. ISS-ODN prepriming effect on the CTL response. The prepriming and immunization schemes used for this study are outlined in Table 2. Mice were sacrificed during wk 8 for determination of splenocyte CTL responses. Results represent the mean $\pm$ SE for four mice in each group, and similar results were obtained in two other independent experiments (13).

Table 3
ISS-ODN Prepriming Effect on Th2 Biased Immune Deviation

OVA/alum	ISS-ODN	IgE (U/ml)	IgG1 (U/ml)	IgG2a (U/ml)
+	None	1682 $\pm$ 421	200 $\pm$ 61	0
+	Day (-1)	139 $\pm$ 139	3201 $\pm$ 885	16077 $\pm$ 7666
+	Day (-3)	0	2098 $\pm$ 489	10078 $\pm$ 2883
+	Day (-7)	147 $\pm$ 147	3810 $\pm$ 420	7183 $\pm$ 460
+	Day (-14)	861 $\pm$ 206	4152 $\pm$ 1274	303 $\pm$ 106

Mice received a single id injection with ISS (50 μ g), 1, 3, 7, or 14 d before OVA (50 μ g) and alum (1mg) via the same route (d 0). Control mice received OVA alum sensitization (Th2) without ISS-ODN. Blood was collected at day 28 for ELISA analysis of antigen specific antibody levels. Results represent the mean $\pm$ SE for four mice in each group and are representative of two similar and independent experiments.

($p < 0.05$) (Table 3), but that ISS-ODN prepriming 14 d prior to sensitization had only a modest effect on the IgE response. In addition, ISS-ODN pre-priming boosted both IgG1 (Th2) and IgG2a (Th1) production ($p < 0.05$) from OVA/alum sensitized mice, while the IgG1/IgG2a response ratio was consistently lower than in sensitized mice that did not receive ISS-ODN. These results demonstrate that ISS-ODN inhibits Th2 biased immune deviation for approximately 7–14 d.

5. DISCUSSION

The data presented demonstrate that id delivery of ISS-ODN up to two wk prior to antigen and into the same site, results in improved antigen specific immune responses when compared to id vaccination with protein alone (8). Antigen specific IgG2a (Th1 isotype) IFN γ , and CTL responses, are all higher in mice preprimed (up to 14 d) with ISS-ODN compared to mice immunized with protein alone. This pre-priming effect does not occur if the interval between ISS-ODN and antigen delivery is extended to 28 d, or if ISS-ODN and antigen are injected into different sites (data not shown). These results further demonstrate that in addition to providing an extended window of local adjuvant activity, id ISS-ODN delivery 3–7 d prior to id protein delivery leads to significantly stronger immune responses than id protein/ISS-ODN coimmunization. We have previously demonstrated that ISS-ODN is an effective mucosal adjuvant (11). In an accompanying review, data is presented that demonstrates that the ISS-ODN prepriming effect also occurs with intra-nasal delivery (Chapter 15). In addition, the antiallergic prepriming potential of ISS-ODN has been explored in protein/alum sensitized mice. These investigations demonstrate that ISS-ODN prepriming prevents the Th2 biased antibody response associated with protein/alum sensitization, while promoting Th1 biased antibody production for 7–14 d.

A previous epidemiological study conducted on approx 1000 Japanese school children demonstrated a correlation between exposure to *Mycobacterium tuberculosis* (MTB) and a Th1 biased serum cytokine profile in study subjects. In addition, PPD converters demonstrated significantly lower serum IgE levels vs PPD negative school children (15). Similar observations have been made in an experimental murine system (16). In this respect, exposure to MTB may be considered to preprime the host toward Th1 immunity. Moreover, a recent study demonstrated that infection of dendritic cells with MTB resulted in the release of TNF α and IL-12 as well as the upregulation of MHC class I, ICAM-1, CD40, and B7 costimulatory molecules (17). This activation profile is very similar to the pattern induced by ISS-ODN (6). As immunostimulatory DNA sequences were initially identified and isolated from MTB DNA (3,18–20), it is conceivable that this DNA adjuvant plays a role in biasing the immune profile of MTB exposed hosts toward a Th1 phenotype, as does synthetic ISS-ODN in mice.

In this review, as well as in recent papers by other investigators, data have been presented that suggest that in gene vaccinated animals, ISS-ODN within the plasmid DNA backbone generate a type 1 cytokine milieu and induce the

expression of costimulatory molecules on APCs and B cells (2, 4–8, 14, 20–24). This activation of the innate immune system would be expected to foster a Th1 response to the antigen encoded in the vaccination plasmid. Thus, gene vaccination plasmids provide both a source of adjuvant and antigen (4, 14, 22, 25, 26). In light of current findings, these two activities are probably not simultaneous. The local induction of cytokines by ISS-ODN is rapid and probably proceeds the expression of sufficient amounts of antigen to elicit an effective immune response. A similar argument can be made for the upregulation of costimulatory ligands. Thus, in gene vaccination, the prepriming effects mediated by immunostimulatory sequences in the pDNA are likely to contribute to the Th1 biased immune response to the encoded antigen.

We present a novel paradigm for Th1 biased immunization that we call ISS-ODN prepriming (8). ISS-ODN administration biases the host immune system toward Th1 biased immune responses for up to 2 wk after exposure. Furthermore, ISS-ODN delivery up to 7 d before antigen produces a stronger immune response than ISS-ODN/antigen coimmunization. Recently, Lipford and colleagues have confirmed that ISS-ODN provides an extended window of Th1 adjuvant activity (27). These findings suggest that ISS-ODN cannot only be used as an adjuvant in the traditional sense, but also as an immunomodifying or immunotherapeutic agent. In murine models, ISS-ODN has been used successfully in the prevention and treatment of *Leishmaniasis*, *Francisella tularensis* and *Listeria monocytogenes* infections (28), indicating that ISS-ODN prepriming induces protective innate immunity to microbial pathogens.

Furthermore, as ISS-ODN prepriming effectively prevents Th2 biased immune dysregulation, it could prove to be a useful intervention for the prevention of allergic hypersensitivities in individuals identified to be at risk for their development. Alternatively, ISS-ODN could be used as an immunotherapeutic agent for patients already suffering from allergic diseases. The prolonged period of type 1 cytokine production induced by ISS-ODN suggests that it is particularly well suited for this kind of allergen independent immunomodulatory therapy. Whereas allergen exposure normally either induces or perpetuates Th2 biased hypersensitivities in allergic patients, during periods of ISS-ODN induced activation of innate immunity, these same exposures could become stimuli for Th1 biased immune responses. Thereby, either preventing or reversing allergic hypersensitivities and protecting against their clinical manifestations. Allergen independent ISS-ODN therapy has already proven highly effective in the attenuation of Th2 biased immune dysregulation and the treatment of asthma and allergic conjunctivitis in murine models (29–31).

Clearly, further research is needed to establish the clinical utility of the ISS-ODN pre-priming effect in the prevention and treatment of infectious and allergic diseases. However, the data generated to date suggest that ISS-ODN has a wide range of potential clinical applications including,

1. Traditional vaccine adjuvant,
2. Immunomodulatory agent for the prevention of infection and allergic hypersensitivities,
3. Immunotherapeutic agent for the treatment of infections and allergic diseases.

ISS-ODN based therapies have been and will likely continue to be studied for safety and efficacy in humans. If ISS-ODN demonstrate similar immunological characteristics in people as it has in mice, it is likely to be a powerful reagent for the prevention and treatment of a wide range of diseases.

ACKNOWLEDGMENTS

We thank Dr. E. Raz for his helpful comments. This work was supported in part by NIH grants AI40682, AI01490, and AI36214, a grant from the Elizabeth Glaser Pediatric AIDS Foundation, and by Dynavax Technologies Corporation.

REFERENCES

1. Ballas, Z. K., Rasmussen, W. L. and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
2. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J. and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
3. Krieg, A. M., Yi, A. K., Matson, S. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
4. Roman, M., E. Martin-Orozco, J. S. Goodman, M. D. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
5. Jakob, T., Walker, P. S., Krieg, A. M., Udey, M. C. and Vogel, J. C. (1998) Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3049.
6. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M. D. Kornbluth, R. S. and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
7. Sparwasser, T., Koch, E. S., Vabulas, R. M. et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.

8. Kobayashi, H., Horner, A. A., Takabayashi, K., et al. (1999) Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity. *Cell Immunol.* **198**, 69–75.
9. Coffman, R. L. and Mosmann, T. R. (1988) Isotype regulation by helper T cells and lymphokines. *Monogr. Allergy* **24**, 96–103.
10. Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J. and Krieg, A. M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
11. Horner, A. A., Ronaghy, A., Cheng, P. M. et al. (1998) Immunostimulatory DNA is a potent mucosal adjuvant. *Cell. Immunol.* **190**, 77–82.
12. Kline, J. N., Waldschmidt, T. J., Businga, T. R. et al. (1998) Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555–2559.
13. Oxenius, A., Martinic, M. M., Hengartner, H. and Klennerman, P. (1999) CpG-containing oligonucleotides are efficient adjuvants for induction of protective antiviral immune responses with T-cell peptide vaccines. *J. Virol.* **73**, 4120–4126.
14. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
15. Shirakawa, T., Enomoto, T., Shimazu, S. and Hopkin, J. M. 1997. The inverse association between tuberculin responses and atopic disorder. *Science* **275**, 77–79.
16. Erb, K. J., Holloway, J. W., Sobeck, A., Moll, H. and Le Gros, G. (1998) Infection of mice with *Mycobacterium bovis*-Bacillus Calmette-Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J. Exp. Med.* **187**, 561–569.
17. Henderson, R. A., Watkins, S. C. and Flynn, J. L. (1997) Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J. Immunol.* **159**, 635–643.
18. Mashiba, H., Matsunaga, K., Tomoda, H., Furusawa, M., Jimi, S. and Tokunaga, T. (1988) In vitro augmentation of natural killer activity of peripheral blood cells from cancer patients by a DNA fraction from *Mycobacterium bovis* BCG. *Jpn. J. Med. Sci. Biol.* **41**, 197–202.
19. Tokunaga, T., Yamamoto, H., Shimada, S., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–962.
20. Yamamoto, S., Kuramoto, E., Shimada, S. and Tokunaga, T. (1988) In vitro augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **79**, 866–873.
21. Halpern, M. D., Kurlander, R. J. and Pisetsky, D. S. (1996) Bacterial DNA induces murine interferon-gamma production by stimulation of interleukin-12 and tumor necrosis factor-alpha. *Cell. Immunol.* **167**, 72–78.
22. Klinman, D. M., Yamshchikov, G. and Ishigatsubo, Y. (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* **158**, 3635–3639.

23. Sun, S., Zhang, X. Tough, D. F. and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Experiment. Med.* **188**, 2335–2342.
24. Tokunaga, T., Yano, O. Kuramoto, E. et al. (1992) Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. *Microbiol. Immunol.* **36**, 55–66.
25. Cohen, A. D., Boyer, J. D. and Weiner, D. B. (1998) Modulating the immune response to genetic immunization. *Faseb J* **12**, 1611–1626.
26. Tighe, H., Corr, M., Roman, M. and Raz, E. (1998) Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today* **19**, 89–97.
27. Lipford, G. B., Sparwasser, T., Zimmermann, S., Heeg, K. and Wagner, H. (2000) CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J. Immunol.* **165**, 1228–1235.
28. Elkins, K. L., Rhinehart-Jones, T. R., Stibitz, S., Conover, J. S. and Klinman, D. M. (1999) Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* **162**, 2291–2298.
29. Broide, D., Schwarze, J. Tighe, H. et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054–7062.
30. Sur, S., Wild, J. S., Choudhury, B. K., Sur, N., Alam, R. and Klinman, D. M. (1999) Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* **162**, 6284–6293.
31. Magone, M. T., Chan, C. C., Beck, L., Whitcup, S. M. and Raz, E. (2000) Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. *Eur. J. Immunol.* **30**, 1841–1850.

Protein-Immunostimulatory DNA-Conjugate

A Novel Immunogen

Kenji Takabayashi, Helen Tighe,
Lucinda Beck, and Hans L. Spiegelberg

1. INTRODUCTION

The original finding made by Tokunaga and colleagues (1,2) demonstrated that DNA from *Mycobacterium bovis* Bacillus Calmette Guérin has antitumor activities and induces natural killer (NK) cells to secrete interferon- γ (IFN- γ). Over the last 10 yr, this finding has stimulated much research on the immunostimulatory properties of bacterial DNA. It was shown that bacterial immunostimulatory DNA sequences (ISS) consist of palindromic sequences of the motif purine-purine-CpG-pyrimidine-pyrimidine (3,4). This motif occurs about 20 times more often in bacterial DNA than in mammalian DNA (5). Furthermore, the cytosine of the crucial CpG sequence in ISS is usually methylated in mammalian DNA and methylated CpG motifs do not show the immuno-stimulatory properties of ISS (6). ISS not only activate NK cells to secrete IFN- γ , but also activate both murine and human antigen presenting cells (APC) to secrete type 1 cytokines such as, IFN- α , β , IL-6, IL-12, and IL-18 (4,7,8), which direct the immune response toward a Th1 immune response. ISS also induce costimulatory molecules on APC (9) and cause human B-cell proliferation and IgM secretion (10). Another property of ISS is the ability to induce “cross-priming,” the phenomenon of priming CD8⁺ cytotoxic lymphocytes (CTL) against foreign protein antigens (11,12). In mice, protein antigens usually induce a predominant Th2 response, which is characterized by the induction of IgG1 and IgE antibodies, and IL-4 and IL-5 secreting T cells. In contrast, when mice were immunized with a protein antigen, together with ISS as the adjuvant, they produced IgG2a antibodies and IFN- γ secreting cells (13,14), which are char-

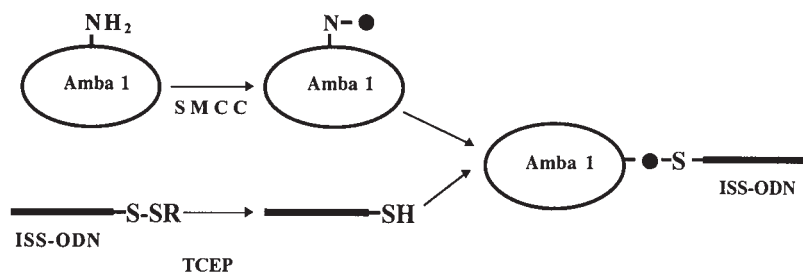
acteristic for a Th1 response. In general, ISS are very strong activators of the innate immune system, and also induce the secretion of tumor necrosis factor- α (TNF- α) and other cytokines that can cause adverse reactions such as septic shock like symptoms both in mice (15,16) and man (17,18). Therefore, the use of ISS as an adjuvant in man may be limited; especially if relatively high doses would be necessary to obtain an adjuvant effect. For this reason, the authors and others investigated ways to obtain maximal ISS effects at a dose that is unlikely to cause general adverse side effects. The authors hypothesized that the adjuvant effect may be optimized if an antigen is covalently coupled to the ISS. This should deliver the antigen and ISS to the same antigen-presenting cell (APC), and allow these cells to give both antigen specific and ISS-mediated signals during the activation of T cells. By injecting antigen conjugated to ISS, the entire antigen would be processed by APCs in the presence of ISS. Free ISS would not be available to systemically activate bystander APCs which produce excess amounts of cytokines that could cause adverse reactions. To test this hypothesis, the authors and others conjugated protein antigens to immuno-stimulatory oligodeoxynucleotides (ISS-ODN) and compared the immune response to such conjugates to that of protein antigens mixed with ISS-ODN (11,12,14,19,20). In all such studies, the protein-ISS conjugates proved to be more immunogenic than the mix of proteins with an amount of ISS-ODN equivalent to that in the conjugates. In this review, the authors summarized data obtained with protein antigen-ISS-ODN conjugates that demonstrate their use for:

1. Inducing strong Th1 and CTL responses (11,14), in particular against the weak antigen gp120 of the human immunodeficiency virus (HIV) (11,21).
2. Inducing Th1 responses to allergens, and down-regulating allergic Th2 responses (19,20).
3. Inducing CTL activity to a tumor specific-antigen that confers resistance to tumor formation in a murine cancer model (12).

2. RESULTS

2.1. Antigen-ISS Conjugates are More Effective than Antigen Mixed with ISS for the Induction of Humoral and Cell-Meditated Immunity

For most studies of the immunogenicity of protein antigen-ISS conjugates, the protein was covalently linked to ISS-ODN by mixing maleimide-activated proteins with thiolated ISS-ODN, a conjugation method that is schematically shown in Fig. 1. However, similar data were obtained by conjugating a thiolated protein with a maleimide-activated ISS-ODN (11), or by physically linking a biotinylated protein and biotinylated ISS-ODN, with



● = Maleimide

TCEP = Tris(2-carboxyethyl)phosphine

SMCC = Succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate

Fig. 1. Schematic representation of the most commonly used conjugation method to prepare protein-ISS-ODN conjugates. First, maleimide groups are incorporated onto the protein, in this example the short ragweed major antigen Amba1, by incubation of the protein with excess sulfoSMCC (sulfosuccinimidyl-4-[N-maleimidomethyl]-cyclohexane-1-carboxylate, Pierce) and excess reagent is removed. Second, a 5'-disulfide modified ISS-ODN is reduced with *tris* (2-carboxyethyl) phosphine, residual reagent removed, and the reduced thio-ISS-ODN incubated with the maleimide treated Amba1 to produce the conjugate. The conjugate is then purified by Superdex HR 200 gel filtration chromatography.

polyvalent avidin (14). The ratio of protein to ISS-ODN varied in different studies from 1:1 to 1:8, however, conjugates of different ratios yielded similar results. These ratios represent the average ISS-ODN molecules bound per molecule of protein because ISS-ODN usually does not conjugate at a single ratio to a protein. This is illustrated for the short ragweed major allergen Amba1 in Fig. 2. When analyzed by SDS-PAGE, Amba1 appears as a single protein band, whereas the conjugate appears as six bands, all having higher molecular weights than Amba1, and all staining for DNA. The multiple bands reflect a heterogeneous coupling of 2 to 6 ISS-ODN molecules per Amba1 molecule, with an average of four ISS-ODN molecules per Amba1 molecule, as determined from the DNA and protein content of the conjugate (19).

Tighe et al. (11) first used thiolated *E. coli* β -galactosidase (β -gal) linked to maleimide activated ISS-ODN as a model antigen. β -gal was used because it allowed for the comparison of β -gal-ISS-ODN conjugate immunization with β -gal gene vaccination, a potent form of immunization, to obtain Th1 and CTL immune responses (22). It was shown that β -gal-ISS-ODN conjugates induced a strong Th1 and CTL response. Mice immunized with such conjugates showed much higher titers of IgG2a anti- β -gal antibodies, and

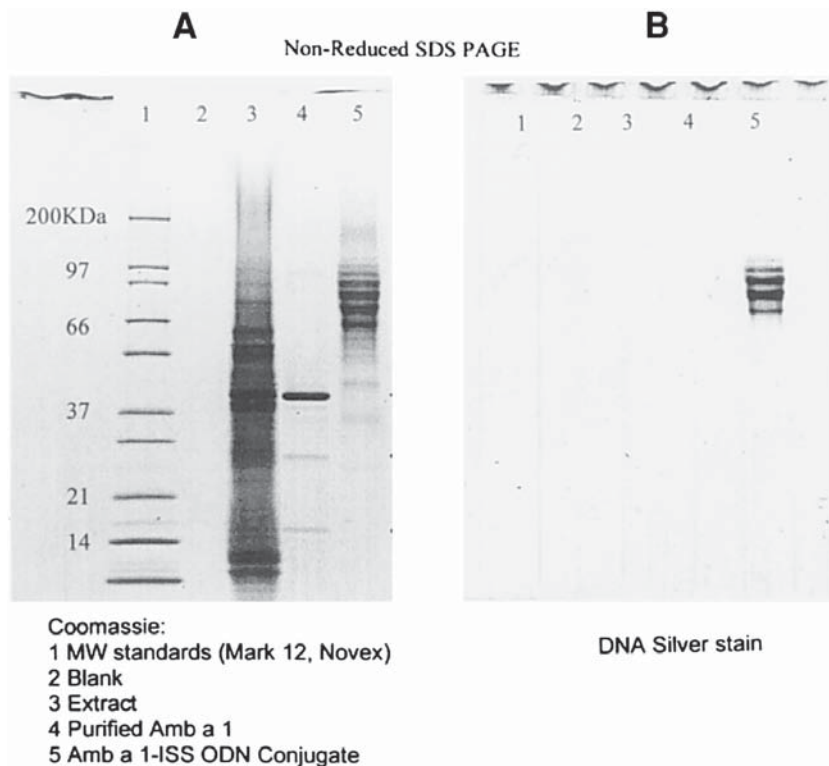


Fig. 2. Nonreduced SDS-PAGE analysis of the Ambal1-ISS-ODN conjugate (gel:4-20%). The protein bands of Ambal1 and Ambal1-ISS-ODN conjugates were visualized with either Coomassie brilliant blue G-250 stain (Fig. 2A) or DNA silver stain (Pharmacia Silver Stain Kit)(Fig. 2B). Lanes: 1, Molecular weight standards; 2, blank; 3, Ragweed pollen extract; 4, purified Ambal1; 5, Ambal1-ISS-ODN conjugate. Figure reproduced from ref. (19) by permission of the *Journal of Allergy and Clinical Immunology*.

lower titers of IgG1 antibodies than mice immunized with a mix of β -gal and ISS-ODN or β -gal alone. The spleen cells of the conjugate immunized mice produced large amounts of IFN- γ and almost no interleukin-5 (IL-5). In contrast, mice injected with β -gal conjugated to a non-ISS-ODN or with β -gal alone formed predominantly IgG1 antibodies, and their spleen cells secreted IL-5, both indicators of Th2 responses. Using biotinylated ovalbumin (OVA) and biotinylated ISS-ODN conjugated with polyvalent avidin, Klinman et al. (14) also showed that conjugation of OVA to ISS-ODN results in a 10-fold increase of total IgG and four-fold increase of IgG2a antibodies, and higher IFN- γ secretion by antigen specific T cells.

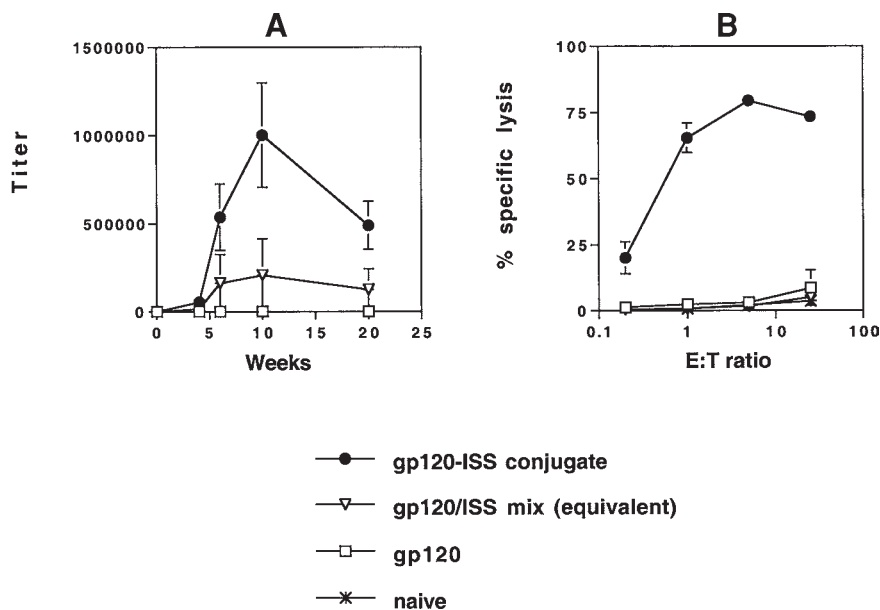


Fig. 3. Induction of A. IgG2a antibodies and B. CTL activity (6 wk after immunization) against the HIV gp120 antigen, by immunization of BALB/c mice with gp120-ISS-ODN conjugate (●), a mix of gp120 and ISS-ODN at amounts equal to those in the conjugates (▽) and gp120 alone (□). (*) = CTL activity of cells from naive mice. Figures are reproduced from ref. 11 by permission of the *European Journal of Immunology*.

β -gal-ISS-ODN conjugates also induced a strong CTL activity. The CTL activity was similar to that observed with gene vaccination, which is known to be a very potent type of immunization for the induction of CTL activity (22,23). In mice, β -gal is a strong immunogen and even β -gal injected in saline induced a low CTL activity. However, as shown in Fig. 3, when the weak human immunodeficiency virus (HIV) glycoprotein antigen, gp120, was studied, only gp120-ISS-ODN conjugates induced high titers of IgG2a antibodies and CTL activity. Immunization with gp120 mixed with an amount of ISS-ODN equal to that in the conjugate, or gp120 alone, did not induce CTL activity (11).

The ability of gp120-ISS-ODN conjugates to induce CTL activity, was further investigated by Horner et al. (21). These authors showed that gp120 mixed with or conjugated to ISS-ODN induced similar amounts of antibody, but that the conjugates induced much more antigen specific IFN- γ secretion by T cells. Gp120-ISS-ODN conjugates also induced secretion of the β -chemokines MIPI- α/β . These chemokines compete with HIV for binding to the CCR5

coreceptor and inhibit HIV cell entry, which would be an additional beneficial effect for a potential gp120-ISS-ODN conjugate vaccine for AIDS.

Gp120-ISS-ODN conjugates were also very potent immunogens when administered intra-nasally (in) to induce mucosal immunity. Whereas intradermal (id) immunization did not induce IgA antibodies, in immunization with gp120-ISS-ODN conjugates induced not only IgA antibodies, but also IgG2a antibodies and IFN- γ secreting T-cells similar to id immunization. The CTL activity induced by gp20-ISS-ODN conjugate was independent of CD4+ T cells since CD4+ T cell depleted mice immunized with gp-120-ISS-ODN conjugates showed the same CTL activity as the normal control mice. Furthermore, spleen cells from mice depleted of CD4+ T cells also formed the same quantities of IFN γ and MIPI- α/β as those of normal mice. Together these properties of gp120-ISS conjugates suggest that they are promising candidates for the design of vaccines for AIDS. The CD4+ T cell independent immune response to gp120-ISS-ODN conjugate is particularly important since AIDS patients have greatly reduced numbers of CD4+ T cells.

2.2. Induction of Th1 Responses and Downregulation of Th2 Responses to Allergens with Allergen-ISS-ODN Conjugates

One of the oldest treatments of allergic diseases is the injection of small but increasing doses of allergen, a therapy that is called allergen specific immunotherapy. Besides clinical improvement of the disease, immunotherapy has also been shown to change the underlying Th2 response to a beneficial Th1 response (24). Although immunotherapy is the only treatment of allergy that changes the underlying pathogenesis of the disease, it is no longer used extensively because the injection of allergen can be associated with potentially life-threatening anaphylactic reactions. Also, the allergen must be repeatedly injected over many years, which is inconvenient for the patient. To determine whether allergen-ISS-ODN conjugates may be more potent and safer immunogens for inducing Th1 responses to allergens and may possibly down-regulate pre-existing Th2 responses to allergens, Tighe et al. (19) studied and compared the immune response in mice to the major short ragweed allergen Amba 1 (Amba1) either conjugated to or mixed with ISS-ODN. Immunization of BALB/c mice with Amba1-ISS-ODN conjugates (AIC) induced IFN γ secreting CD4+ T cells and high titers of IgG2a but no IgE antibodies, all indicators of a Th1 response. In contrast, Amba1 when injected in saline and particularly when injected in alum, induced IgG1 and IgE antibodies and IL-5 secreting T cells, all of which are indicative of Th2 responses. Amba1 conjugated to a non-ISS-ODN did not induce a Th1 immune response to Amba1. When Amba1 was injected mixed with an

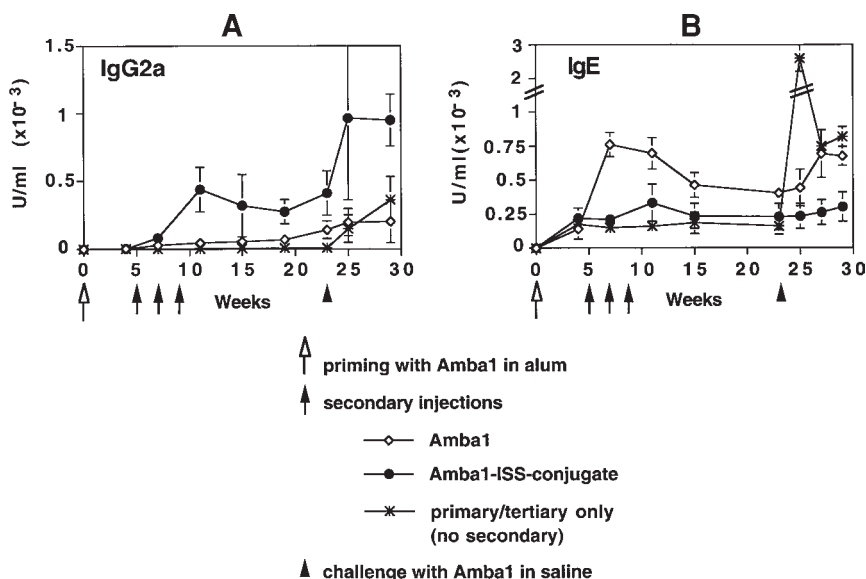


Fig. 4. Effect of secondary immunization (“treatment”) with Amba1-ISS-ODN conjugate (●), Amba1 in saline (◇) or saline alone (✱) on IgG2a and IgE antibody formation after a tertiary challenge injection of 10 μ g Amba1 in saline 13 wk after the last secondary “treatment” injection. Mean $\pm$ SEM of 4 BALB/c mice. Figures are reproduced from ref. 19 by permission of the *Journal of Allergy and Clinical Immunology*.

amount of ISS-ODN equal to that in the conjugate, there was a 3- to 4-fold lower IgG2a antibody response and 10-fold lower IFN γ secretion by CD4 $^{+}$ T cells, as compared to AIC injected mice. AIC also induced higher titers of IgG antibodies than Amba1 mixed with ISS-ODN when injected in rabbits and cynomolgus monkeys (19).

We then studied whether pre-existing Th2 and IgE antibody responses could be down-regulated by AIC immunization (19), a situation which is clinically more relevant than studying the primary immune response to allergens since by definition allergic patients have preexisting Th2 anti-allergen responses before treatment is initiated. For this purpose, mice were first immunized with Amba1 in alum and then given three bi-weekly injection of AIC (Fig. 4). Control mice received either three bi-weekly injections of saline alone or Amba1 in saline. During this “treatment” period, only the mice injected with AIC formed increasing amounts of IgG2a antibodies without a concomitant increase of the IgE titer. In contrast, the IgE titer increased in the Amba1 injected control mice and remained unchanged in

the saline control group. After a rest period of 14 wk, the mice were challenged with a tertiary injection of 10 μ g Amba1 in saline. This induced a rise in the IgG2a titer, but no significant rise in the IgE titer in the AIC treated mice. In contrast, the saline injected control mice showed a large increase in the IgE titer and only a very small rise in the IgG2a titer. The Amba1 injected control mice showed an increase of the IgE and IgG2a antibody titers that were intermediate to those of the AIC and saline injected mice. The mice were then sacrificed and the IFN- γ and IL-5 secretion of the CD4+ T cells in the spleen was determined. The cells of the AIC treated mice secreted significantly more IFN γ and less IL-5 than the cells from the control groups. These data show that a preexisting Th2 response to the Amba1 allergen did not prevent the induction of a new Th1 anti-Amba1 response, as shown by the large increase of the IgG2a antibody titer and IFN γ secretion by the CD4+ spleen cells. Furthermore, "treatment" with AIC inhibited the IgE antibody booster response after the tertiary Amba 1 injection. "Treatment" with Amba 1 in saline, which was analogous to immunotherapy in man, only partially lowered the IgE response to the tertiary Amba1 injection and did not induce IFN- γ secretion. This showed in mice that AIC immunization was superior to Amba1 immunization for down-regulating a preexisting Th2 anti-allergen response.

Equally important was the observation that AIC not only were more immunogenic for inducing Th1 and IgG responses than Amba1 mixed with ISS-ODN but also were less allergenic in vitro in two human systems (19). First, AIC were less reactive with human IgE antibodies than native Amba1 in an ELISA inhibition test. Second, about 100-fold more AIC than Amba1 was necessary to induce the same amount of histamine release from basophils of ragweed allergic patients.

In summary, chemical conjugation of Amba1 to ISS-ODN conferred on the allergen two new properties that make it an excellent candidate for human immunotherapy. First, AIC rapidly induced a Th1 antiallergen response in mice as indicated by IgG2a, but no IgE antibody formation and by robust IFN- γ secretion by allergen activated CD4+ spleen cells. This Th1 response was induced even in mice having a preexisting anti-Amba1 Th2 response, and, over time, AIC downregulate this Th2 response. Second, AIC are less allergenic than native allergen or allergen mixed with ISS-ODN. Based on the in vitro data with human IgE antibodies and basophils, one might expect that ragweed allergic patients could tolerate the injection of larger quantities of AIC than Amba1. Indeed, in the first clinical trials with AIC in ragweed sensitive patients, equally positive wheel and flare skin reactions were obtained only by injecting 200-fold more AIC than Amba1 (25).

2.3. Effect of OVA-ISS-ODN Conjugates on Airway Eosinophilia and Hyperresponsiveness in a Mouse Model of Asthma and Mechanism of APC Activation by Protein-ISS-ODN Conjugates

Shirota et al. (20) studied the effect of intratracheally administered OVA-ISS-ODN conjugates on airway eosinophilia and hyperresponsiveness in a mouse model of asthma. They showed that the conjugate was 100-fold more efficient than a mix of OVA and ISS-ODN in reducing the airway eosinophilia and airway hyperresponsiveness. This effect lasted for more than two months. The decrease of airway eosinophilia and hyperresponsiveness was accompanied by a decrease of IL-4 and IL-5 secretion, and a 100-fold increase in the IFN- γ secretion by OVA activated lymph node T cells, indicating that the OVA-ISS-ODN conjugates converted the anti-OVA Th2 response to a Th1 response. Because this intratracheal administration of OVA-ISS-ODN conjugates was so effective in reducing two parameters in the mouse model of asthma, the authors suggested that allergen-ISS-ODN conjugates also might be useful for inhalation immunotherapy for the treatment of asthma in man.

More recently, Shirota et al. (26) reported interesting data on the mechanism of activation of dendritic cells (DC) by allergen-ISS-ODN conjugates. These investigators studied the bindings of fluoresceinated OVA and OVA-ISS-ODN conjugates to DC. They showed that OVA-ISS-ODN conjugates bound to higher percentage of DC and at higher fluorescence intensity, as compared to OVA. The augmented bindings of the conjugates lead to a greater uptake of the conjugates into endosomes and cytoplasm of the DC than of OVA alone. This finding was true not only for OVA-ISS-ODN conjugates, but also for phycobiliprotein-ISS-ODN conjugates that also bound to a higher percentage of DC, and with a higher intensity than the native protein as well as endocytosed faster than native phycobiliprotein. After phagocytosis of the conjugates, DCs incubated with conjugates showed an increase of costimulatory molecules and IL-12 secretion, as compared to DCs incubated with OVA alone. Therefore, it was concluded that ISS-ODN conjugated to antigen act as promoters of antigen phagocytosis and subsequent cell activation. Conjugates prepared with a non-ISS-ODN bound to DC the same as ISS-ODN conjugates, but did not activate the cells for IL-12 secretion. Therefore, binding and phagocytosis of the conjugates does not appear to be ISS-ODN specific, however, the subsequent cell activation and cytokine secretion was ISS specific. Whether the Toll-like receptor 9 (TLR-9), which appears to be the ISS-DNA receptor on APC (27,28), was involved in the binding of the conjugates to DC was not investigated in these experiments and remains to be shown. However, the data demonstrate that conju-

gation of ISS-ODN to a protein antigen enhances the binding to and phagocytosis of that protein by the APC resulting in increased APC activity. This represents at least one of the mechanisms responsible for the enhanced immunogenicity of antigen-ISS-ODN conjugates.

2.4. Protein-ISS-ODN Vaccines for Induction of CTL and Protection of Tumor Growth

Cho et al. (12) studied the effect of OVA-ISS-ODN conjugate immunization on prevention and retardation of tumor growth in a mouse model of cancer. These authors used a transgenic EL-4 thymoma cell line that expresses OVA as the tumor specific antigen (E.G7-OVA). Preimmunization with OVA-ISS-ODN conjugates prevented the *in vivo* growth of E.G7-OVA cells, but not the growth of wild-type (WT) EL-4 cells. Neither immunization with OVA alone, nor OVA conjugated to a non-ISS-ODN, nor ISS-ODN alone conferred the tumor protection. When different immunization procedures were compared, OVA-ISS-ODN conjugate immunization was the most efficient in inducing CTL and preventing specific tumor growth. OVA injected mixed with ISS-ODN or OVA gene immunization induced lower CTL activity. OVA-ISS-ODN conjugate immunization not only prevented tumor growth, but also induced regression of already established tumors. Interestingly, the induction of tumoricidal activity by the conjugates was independent of CD4⁺ T cells because it could be induced equally well in CD4^{-/-} knockout (KO) and WT control mice. CD8⁺ CTL were responsible for the tumoricidal activity because OVA-ISS-ODN conjugate immunization did not prevent tumor growth in CD8^{-/-} KO mice. The induction of CTL was not only independent of CD4⁺ T cells, but also was independent of MHC class II molecules because it occurred similarly in MHC class II^{-/-} KO and WT control mice. These data show that, at least in this model of cancer, conjugates of ISS-ODN to a tumor specific antigen-induced tumor protective CTL in a CD4⁺ T cell independent manner. Although the abilities of such conjugates to protect from naturally occurring tumors remains to be shown, the data encourage the testing of their application for human tumors.

3. DISCUSSION

Chemical conjugation via a maleimide-sulfide bridge (11,19,20) or physical conjugation via biotin and avidin (14) of a protein antigen to ISS-ODN greatly enhances the immunogenicity of the protein antigen, as compared to immunization with a mix of antigen and ISS-ODN. This has been shown in mice for:

1. Several proteins, and was especially true for a weak antigen, the HIV gp120 envelope protein (11,21)
2. For the allergen Ambal (19).
3. For OVA as the tumor specific antigen in a mouse model of cancer (12).

The mechanism of the enhanced immunogenicity of protein-ISS-ODN conjugates is likely owing to the delivery of both antigen and ISS-ODN to the same APC. If injected as a conjugate, all of the antigen is processed by the APCs under the influence of the ISS-ODN and subsequently all deliver both the antigen and ISS induced signals to the T cells during T cell activation. Second, protein-ISS-ODN conjugates bind more strongly to APC such as DC (26) presumably by binding to ISS receptors, which leads to enhanced phagocytosis of the antigen and APC activation by the ISS-ODN. These and possibly other factors are likely to be responsible for the maximal immune responses obtained with low doses of antigen-ISS-ODN conjugates and thus, diminishes the possibility of ISS-ODN induced general adverse reactions resulting from excess cytokine secretion, which could occur when antigen ISS-ODN mixes are used (15,16,18). The enhanced immunogenicity and presumably lower toxicity of antigen-ISS-ODN conjugates make them promising novel candidates for the development of vaccines for human use.

The possible clinical applications of antigen-ISS-ODN conjugates were shown in three preclinical mouse models. First, it was demonstrated that conjugation of the weak HIV gp120 antigen becomes very immunogenic and induces a CTL response when conjugated to an ISS-ODN. Mice immunized with gp120-ISS-ODN conjugates formed high titers of IgG2a antibodies and their T cells secreted large amounts of IFN γ . Most importantly, the conjugate induced CTL activity was CD4+ T-cell independent, a feature which is very important for designing AIDS vaccines since AIDS patients have very low numbers of CD4+ T helper cells. Therefore, a gp120-ISS-ODN vaccine may induce CTL in AIDS patients. In a model of allergy, short ragweed allergen Ambal-ISS-ODN conjugates induced a Th1 anti-Ambal response as indicated by IFN- γ secreting T cells and IgG2a but no IgE antibody formation. This Th1 response also was induced in mice having a preexisting Th2 response and downregulated the IgE antibody formation to a tertiary immunization with Ambal. In addition, OVA-ISS-ODN immunization reduced symptoms of asthma in a mouse model (20) with OVA induced airway eosinophilia and hyper-responsiveness being greatly reduced after immunization with OVA-ISS-ODN conjugates. Conjugation of ISS-ODN to Ambal had another additional beneficial effect in its possible use for allergen immunotherapy. Conjugation of an average of four ISS-ODN molecules to one Ambal molecule greatly reduced its allergenicity, as

shown by lower reactivity with human IgE antibodies and lower histamine release from basophiles of ragweed allergic patients. This suggested that greater amounts of allergen in the form of conjugates are likely to be tolerated by patients undergoing conjugate immunotherapy. Indeed, this was the case, in the first clinical trials of Amba1-ISS-ODN conjugates (25). Third, OVA-ISS-ODN immunization prevented the growth and caused regression of already established tumors that expressed OVA on the surface as a tumor specific antigen. Together, all these preclinical data of antigen-ISS-ODN activity strongly suggest that they are excellent candidates for the development of more effective vaccines in man. The first data of phase I/II trials of Amba1-ISS-ODN conjugates for the treatment of patients with allergic rhinitis in response to short ragweed pollen appears to prove this notion.

Moreover, it is unlikely that immunization with antigen-ISS-ODN conjugates induce adverse reactions in man. First, only small amounts of ISS-ODN in form of conjugates will likely be necessary to obtain maximal immune responses and all of the ISS-ODN will be bound to antigen. This should minimize a possible excess production of cytokines such TNF- α that could cause adverse reactions. Second, no antidouble-stranded DNA antibodies were detected after injection with antigen-ISS-ODN conjugates in mice, as well as in man during phase I trials. Although more clinical trial will have to be performed to determine the effectiveness of antigen-ISS-ODN conjugates, all data obtained to date indicate that the conjugates are likely to be potent and safe candidates for the development of more efficient vaccines for a variety of human conditions.

ACKNOWLEDGMENTS

We thank Dr. E. Raz for his support and helpful comments. This work was supported by NIH program project grant no. AI 40682 and AI 47078, and by Dynavax Technologies Incorporation.

REFERENCES

1. Tokunaga, T., Yamamoto, H., Shimada, S., et al. (1984) Antitumor activity of the deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–962.
2. Yamamoto, S., Kuramoto, E., Shimada, S., and Tokunaga, T. (1988) *In vitro* augmentation of natural killer cell activity and production of interferon- α/β and - γ with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **79**, 866–873.

3. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.
4. Klinman, D. M., Yi, A., Beaucouge, L., Comover, J., and Krieg, A. M. (1996) CpG motifs presenting bacterial DNA rapidly induce lymphocytes to secrete interleukin-6, interleukin-12, and interferon- γ . *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
5. Bird, A. P. (1987) CpG islands as gene markers in the vertebrate nucleus. *Trends Genet.* **3**, 342–347.
6. Yamamoto, S., Yamamoto, T., Shimada, S., et al. (1992) DNA from bacteria, but not from vertebrates, induces interferon, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* **36**, 983–997.
7. Halpern, M. D., Kurlander, R. J., and Pisetsky, D. S. (1996) Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell. Immunol.* **167**, 72–78.
8. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and INF- γ production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* **29**, 2344–2353.
9. Martin-Orosco, E., Kobayashi, H., Van Uden, J., Nguyen, M.-D., Kornbirth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
10. Krieg, A. M., Yi, A.-K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
11. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947.
12. Cho, H. J., Takabayashi, K., Cheng, P.-M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nature Biotech.* **18**, 509–514.
13. Roman, M., Martin-Orosco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
14. Klinman, D. M., Barnhar, K. M., and Conover, J. (1999) CpG motifs as immune adjuvants. *Vaccine* **17**, 19–25.
15. Sparwasser, T., Miethke, T., Lipford, G., et al. (1997) Macrophages sense pathogens via DNA motifs: induction of TNF- α -mediated shock. *Eur. J. Immunol.* **27**, 1671–1677.
16. Lipford, G.B., Sparwasser, T., Bauser, M., et al. (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* **27**, 3420–3426.
17. Bohle, B., Orel, L., Kraft, D., and Ebner, C. (2001) Oligodeoxynucleotides containing CpG motifs induce low levels of TNF- α in human B lymphocytes: possible adjuvants for Th1 responses. *J. Immunol.* **166**, 3743–3756.

18. Michie, H. R., Manogue, K. R., Spriggs, D. R., et al. (1988) Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* **318**, 1481–1485.
19. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* **106**, 124–134.
20. Shiota, H., Sano, K., Kikuchi, T., Tamura, G., and Shirato, K. (2000) Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J. Immunol.* **164**, 5575–5582.
21. Horner, A. A., Datta, S. K., Takabayashi, K., et al. (2001) Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. *J. Immunol.* **167**, 1584–1591.
22. Raz, E., Carson, D. A., Parker, S. E., et al. (1994) Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* **91**, 9519–9523.
23. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
24. McHugh, S. M., Deighton, J., Stewart, A. G., Lachman, P. J., and Ewan, P. W. (1995) Bee venom immunotherapy induces a shift in cytokine response from a TH-2 to a TH1-1 dominant pattern: comparison of rush and conventional immunotherapy. *Clin. Exp. Allergy* **25**, 828–838.
25. Creticos, P. S., Eiden, J. J., Balcer, S. L., et al. (2000) Immunostimulatory oligodeoxynucleotides conjugated to Amb a 1: safety, skin test reactivity, and basophil histamine release. *J. Allergy Clin. Immunol.* **105**, S70.
26. Shiota, H., Sano, K., Hirasawa, N., et al. (2001) Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. *J. Immunol.* **167**, 66–74.
27. Hemmi, H., Takkeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
28. Wagner, H. (2001) Toll meets bacterial CpG-DNA. *Immunity* **14**, 499–502.

Immunostimulatory DNA Sequence Based Mucosal Vaccines

Anthony A. Horner

1. INTRODUCTION

Unlike the skin, which is relatively impermeable, mucosal surfaces allow for the intake of oxygen, water, and other nutrients, and the excretion of metabolic by-products. This relatively permeable nature places unique constraints and requirements on the immunocytes, which protect mucosal surfaces, and makes these sites particularly susceptible to penetration by infectious agents. Despite the relative efficiency of the mucosal immune system in preventing infection, the respiratory, gastrointestinal, and vaginal mucosa represent major portals of entry for pathogens (1,2). Locally produced and secreted IgA and cytotoxic T lymphocytes (CTL) within the mucosal tissue and draining lymph nodes protect against microbial infection (1–4). Because the mucosal immune system serves a front line role in protecting us from potential pathogens, there is a great deal of interest in developing strategies for stimulating protective mucosal immune responses. Live viral vaccines, such as the attenuated oral polio vaccine, induce strong mucosal immune responses. However, only a limited number of attenuated vaccines exist. The majority used in clinical practice are derived from recombinant proteins (2). These protein based vaccines tend to induce humoral but not cellular immunity when injected systemically, and mucosal delivery often leads to tolerance. Here we review recent investigations, which establish that immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) as an effective mucosal adjuvant that can be used to generate both humoral and cellular immunity to a wide range of antigens, both systemically and in mucosal tissue (5–9).

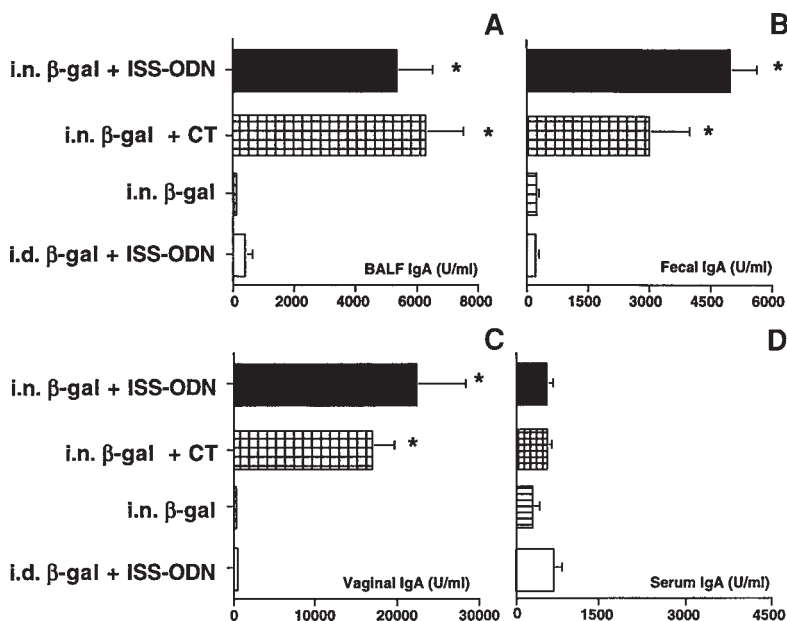


Fig. 1. IgA responses. Mice were immunized with β -gal(50mg) alone, or with ISS-ODN (50 μ g), or CT (10 μ g) via the i.n. or i.d. route on a single occasion. The ISS-ODN used in these experiments has the sequence 5'-TGACTGTGAACGTTTCGAGATGA-3'. Immunization, sample collection, and ELISA methods are described in ref (5). Results were obtained by ELISA analysis of samples collected during wk 7, and represent mean values for 4 mice per group. Error bars reflect the standard errors of the means. Results are representative of 3 similar and independent experiments (5). Mucosal IgA levels of i.n. β -gal/ISS-ODN and i.n. β -gal/CT immunized mice, were similar and significantly higher than control immunized mice (* $p < 0.05$). (A) BALF IgA. (B) Fecal IgA. (C) Vaginal IgA. (D) Serum IgA. Serum was obtained at wk 7.

2. INTRANASAL VACCINATION WITH ISS-ODN ELICITS SECRETORY IGA RESPONSES AT LOCAL AND DISTAL SITES

As a proof of principle, we intranasally (i.n.) immunized mice with ISS-ODN and an experimental antigen (β -galactosidase; β -gal). Cholera toxin (CT), the best-studied and most potent known mucosal adjuvant was used as a control (2). In Fig. 1, the mucosal IgA responses of BALB/c mice i.n. immunized with β -gal and ISS-ODN or CT are presented. Although direct comparison of the IgA levels from different mucosal sites cannot be made because of differences in sample collection techniques, the data clearly demonstrates that β -gal/ISS-ODN and β -gal/CT immunized mice had equiva-

lent antigen-specific IgA levels in bronchial, intestinal, and vaginal secretions. To establish that a mucosal adjuvant was needed for the induction of mucosal IgA, mice were i.n. immunized with β -gal alone or with a mutated oligodeoxynucleotide (M-ODN). However, i.n. vaccination without an adjuvant resulted in no detectable IgA in mucosal secretions (data for i.n. β -gal immunization with M-ODN not shown). Furthermore, Mucosal delivery was required for ISS-ODN to elicit a mucosal IgA response, as i.d. β -gal/ISS-ODN immunized mice did not have IGM in their secretions. The IgA detected in the mucosal secretions of i.n. immunized mice appeared to be owing to active secretion and not passive diffusion, as IgA levels were consistently higher in mucosal vs serum samples, despite the dilution required to determine fecal, vaginal, and bronchoalveolar lavage fluid (BALF), but not serum IgA levels. These results show that ISS-ODN and CT have equivalent mucosal adjuvant activity for the generation of secretory IgA, with a test antigen that has no capacity to induce mucosal IgA production when delivered alone. In addition, i.n. but not i.d. delivery of antigen with ISS-ODN is demonstrated to elicit a secretory IgA response. Taken together, these findings demonstrate that ISS-ODN is an excellent adjuvant for the induction of both local and distal mucosal IgA responses, when codelivered with antigen via the nose.

2.1. Mucosal Immunization with ISS-ODN Leads to a Th₁ Biased Antibody Response

Next evaluated was the serum antibody response induced by i.n. immunization with ISS-ODN. Again, the mucosal adjuvant CT was used for comparison. As can be seen in Fig. 2, i.n. vaccination with ISS-ODN and β -gal led to high serum levels of IgG2a (IFN γ dependent), and low serum levels of IgG1 (IL-4 dependent), compared to i.n. β -gal/CT vaccination. In addition, β -gal/CT, but not β -gal/ISS-ODN immunized mice produced antigen specific IgE. Furthermore, the serum antibody profiles induced by i.n. and i.d. delivery of β -gal and ISS were found to be equivalent in magnitude and Th bias, suggesting that ISS-based vaccines delivered by these routes have similar efficacy for the induction of systemic immunity. Again, the requirement for i.n. immunization with an adjuvant was demonstrated as i.n. delivery of β -gal alone, or with M-ODN, did not induce a serum antibody response (data for i.n. β -gal/M-ODN delivery not shown) .

2.2. Mucosal Immunization with ISS-ODN Induces an Antigen-specific IFN γ , IL-6, and IL-10 Response

The adjuvants ISS-ODN and CT have previously been considered to promote antigen specific Th₁ and Th₂ biased cytokine profiles, respectively (5,7,10–12). To determine whether i.n. and i.d. β -gal and ISS-ODN

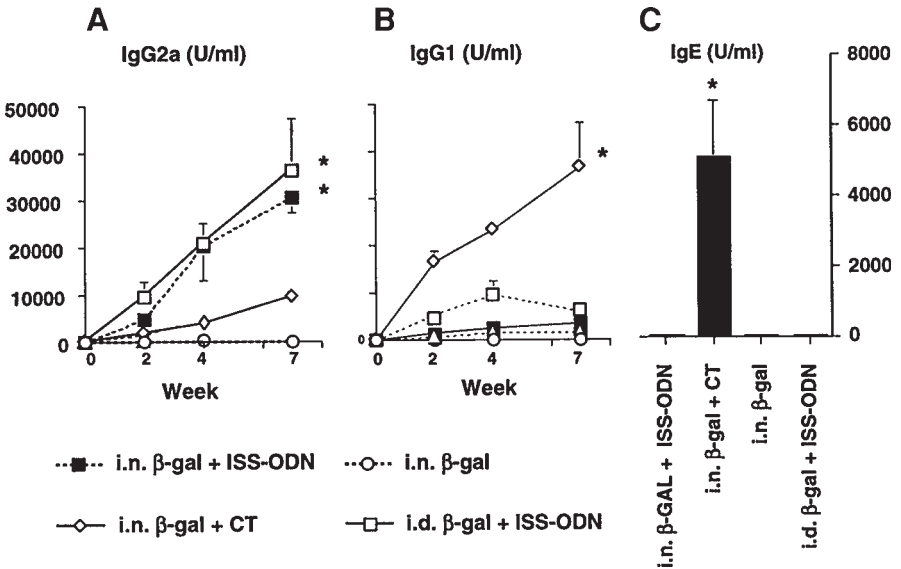


Fig. 2. Serum IgG subclass profiles and IgE. Mice were immunized as described in Fig. 1. Serum was collected at seven weeks from immunized mice and assayed by ELISA. Results represent mean values for four mice per group, and error bars reflect standard errors of the means. Results are representative of three similar and independent experiments (5). Significance differences between i.n. β-gal/ISS-ODN vs i.n. β-gal/CT immunized mice are identified with a symbol (* $p \leq 0.05$). (A) Serum IgG2a. (B) Serum IgG1. (C) Serum IgE.

immunization induced similar Th₁ biased cytokine responses, splenocytes from immunized mice were incubated with β-gal, and culture supernatants assayed for the production of IFNγ and IL-4. Splenocytes from mice immunized with β-gal and ISS-ODN by either the i.n. or i.d. route, produced equivalent and high levels of IFNγ (Fig. 3), but no IL-4. Whereas splenocytes from i.n. β-gal/CT immunized mice produced IL-4, and only low levels of IFNγ. Splenocytes from mice i.n. immunized with β-gal alone or with M-ODN, did not demonstrate any detectable antigen-specific cytokine production, further demonstrating the need for an adjuvant in the induction of productive immune responses at mucosal sites.

IFNγ has not been shown to induce mucosal IgA production. However, ISS-ODN elicits antigen independent production of IL-6 and IL-10, both of which facilitate IgA synthesis (13–20). Therefore, we evaluated next, the antigen-specific IL-6 and IL-10 responses of immunized mice. Splenocytes

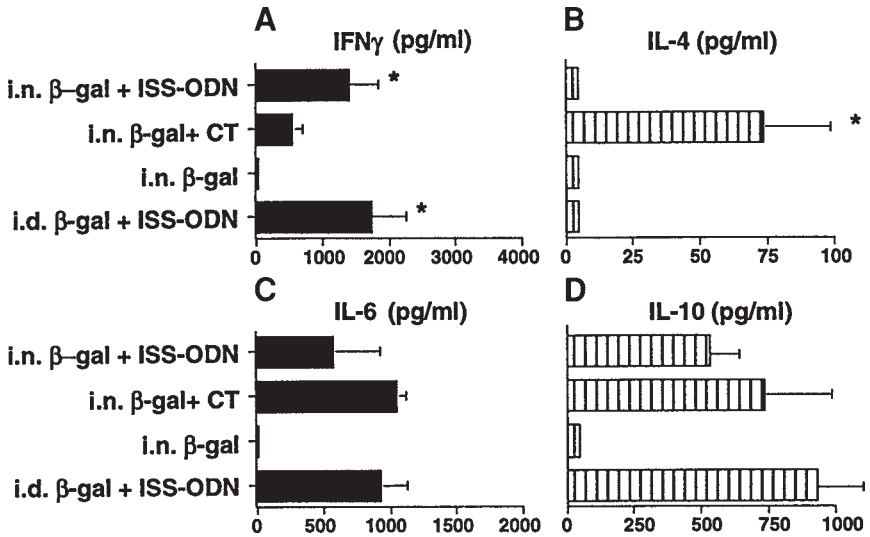


Fig. 3. Antigen induced splenocyte cytokine profiles. Mice were immunized, as described in Fig. 1. Splenocytes were harvested from sacrificed mice during wk 7, cultured in media with or without β -gal (10 μ g/mL), and 72-h supernatants were assayed by ELISA. Splenocytes from immunized mice cultured without β -gal produced negligible amounts of cytokines (data not shown). Results represent the mean for four mice in each group, and similar results were obtained in two other independent experiments (5). Error bars reflect standard errors of the means. Intranasal and intradermal immunization with β -gal and ISS-ODN led to significantly higher IFN γ and lower IL-4 production than i.n. β -gal/CT vaccination (* $p \leq 0.05$). (A) IFN γ . (B) IL-4. (C) IL-6. (D) IL-10.

from mice i.n. and i.d. immunized with β -gal and ISS-ODN and splenocytes from i.n. β -gal and CT immunized mice produced significant and equivalent levels of IL-6 and IL-10 (Fig. 3). In addition, IL-6 and IL-10 were produced by lymphocytes residing in the lung parenchyma of i.n. β -gal/ISS-ODN and i.n. β -gal/CT immunized mice. However, pulmonary lymphocytes from mice i.n. immunized with β -gal alone, produced only low levels of IL-6 and no detectable IL-10 (data not shown).

2.3. Mucosal Immunization with ISS-ODN Induces a Splenic CTL Response

Although development of antigen-specific CTL activity is associated with Th₁ biased immunity, not all Th₁ biased immune responses include the development of cytotoxic T cells (2,3). Therefore, the ability of i.n. codelivery of β -gal and ISS-ODN to induce a CTL response was evaluated.

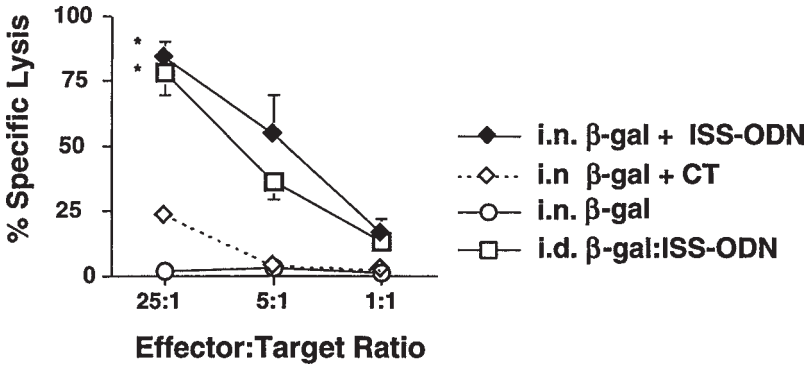


Fig. 4. Splenic CTL responses. Mice were immunized as described, I Fig. 1. Results were obtained by ELISA using a substrate that is metabolized by LDH from lysed cells to a colored byproduct. Results represent mean values for four mice per group, and Error bars reflect the standard errors of the means (5). Results are representative of three similar and independent experiments. The CTL responses induced by i.n. β -gal/ISS-ODN and i.d. β -gal/ISS-ODN immunizations were not significantly different, but were significantly more robust then the CTL elicited by i.n. β -gal/CT immunization(* $p \leq 0.05$).

As demonstrated in Fig. 4, mice immunized with β -gal and ISS-ODN, by either the i.n. or i.d. route, displayed vigorous and equivalent splenic CTL activity. Again, i.n. immunization with β -gal alone, or with M-ODN, led to poor or undetectable CTL responses (data for i.n. β -gal/M-ODN immunization not presented). Considered in conjunction with the splenic cytokine and serum antibody responses previously presented, this data confirms that i.d. and i.n. vaccination with β -gal and ISS-ODN lead to systemic immune responses of equivalent magnitude.

2.4. Mucosal ISS-ODN Prepriming

We have recently found that i.d. ISS-ODN injection of mice induces innate immune activation for an extended period of time (21,22). Therefore, we considered the possibility that pre-priming with ISS-ODN would also lead to an extended period of adjuvant activity for the generation of Th1 biased adaptive immune responses. Work by Kobayashi and colleagues demonstrates that in fact, i.d. injection of ISS-ODN up to 2 wk before β -gal delivery increases the magnitude of immune response compared to immunization with β -gal alone (22). Furthermore, ISS-ODN prepriming 3–7 d before antigen delivery leads to a more robust immune response than coadministration. In conjunction with these studies, we have found that there is a 7 d window of adjuvant activity after i.n. ISS-ODN delivery, with

Table 1
Anti-β-gal Ig Production Induced by Mucosal Prepriming

ISS-ODN	β-gal	IgG2a (U/ml)	IgA (U/ml)	IFNγ (pg/ml)	% Specific lysis (25:1 E:T ratio)
None	+	<5	<50	106±28	5±2
Day (0)	+	2390±715*	2940±825*	1056±123*	82±13*
Day (-1)	+	3220±1120*	463±47*	13642±4283*	81±23*
Day (-3)	+	380±34*	398±38*	4630±210*	76±21*
Day (-7)	+	178±48*	459±183*	506±101*	51±14*
Day (-14)	+	<5	<50	264±53	7±3

Mice received i.n. ISS-ODN (50μg) on a single occasion. the specified number of days before or with i.n. β-gal (50μg). The sequence of the oligodeoxynucleotide used in these experiments is provided in the Fig. 1 legend. Serum and BALF were obtained at wk 8 and analyzed for Ig content, as previously described (22). Splenocytes from immunized mice were cultured with β-gal (72 h) or class I restricted β-gal peptide (5 d), and IFNγ levels and CTL activity were measured, as previously described (22). Ig levels, cytokine, and CTL responses from mice receiving ISS-ODN with or up to 7 d before β-gal were significantly higher then those from mice receiving β-gal alone (**p* < 0.05) (22).

improved immune responses seen if ISS-ODN is given 1–3 d before ISS-ODN (Table 1). The mechanisms underlying ISS-ODN prepriming are discussed in detail in Dr. Kobayashi’s prepriming review.

2.5. ISS-ODN is an Effective Mucosal Adjuvant for a Wide Range of Protein Antigens via Several Vaccination Routes

To further investigate its effectiveness as a mucosal adjuvant, the authors i.n. immunized several strains of mice with ovalbumin (OVA) and ISS-ODN. Consistent with studies outlined previously, both systemic and mucosal OVA specific immunity was found to develop in i.n. OVA/ISS-ODN immunized BALB/c, C57Bl/6, and C3H/HeJ mice (unpublished data). Moldoveanu et al. have gone on to show that i.n. vaccination, with formalin inactivated influenza vaccine and ISS-ODN, also leads to robust mucosal IgA and serum IgG responses (6). Interestingly, under the experimental conditions utilized in these studies, i.n. delivery of the influenza vaccine without adjuvant elicited low level immunity. The mutimeric nature of the antigen preparation, and the epithelial binding properties of the influenza virus may help explain this observation (23). However, these investigators were unable to detect a CTL response against influenza virus. Formalin inactivation leads to crosslinking of protein. This in turn could limit peptide processing and presentation by MHC molecules, leading to relatively weak T-cell responses as suggested by the authors (6,23). McCluskie and colleagues have also recently shown that ISS-ODN serves as an effective

mucosal adjuvant for the induction of mucosal IgA and serum IgG responses against hepatitis B surface antigen and tetanus toxoid (7,8). Unlike the influenza vaccine, but consistent with antigens used in our studies, these antigens did not induce an immune response when mucosally delivered without adjuvant. Gallichan has gone on to demonstrate that i.n. delivery of an ISS based HSV-2 vaccine protects against vaginal challenge with virus (24).

McCluskie's research group has also recently shown that in addition to its utility for i.n. vaccination, ISS-ODN is an effective adjuvant for oral and rectal immunization (8). Interestingly, in our own studies, ISS-ODN was a poor adjuvant for oral vs i.n. vaccination. However, we orally immunized mice on a single occasion, whereas McCluskie's group delivered oral antigen and ISS-ODN on multiple occasions. These observations suggest that ISS-ODN is an effective adjuvant for immunization via a variety of mucosal routes, but that the nasal route may be superior when antigen and ISS-ODN dosing are limited. In consideration of the acidic pH and enzymatic activity found in the stomach, oral immunization with ISS-ODN might be further improved by procedures, such as lipid encapsulation to protect the immunization reagents from degradation.

Recently, studies have established that physical conjugates of antigen and ISS-ODN have significantly improved immunogenic potential when compared to antigen mixed with ISS-ODN (*see* Chapters 14 and 19) (25,26). Based on these observations, we have conducted a series of experiments vaccinating mice with a physical conjugate of gp120 from HIV and ISS-ODN (gp120:ISS-ODN). These investigations establish that gp120:ISS-ODN is a potent immunogen for both i.d. and i.n. vaccination, even though gp120 induces only a weak immune response when delivered alone by either immunization route (9). Consistent with other investigations from this laboratory, these studies further establish that gp120:ISS-ODN vaccination elicits a stronger and more consistent immune response than gp120 mixed with much higher doses of ISS-ODN. As discussed in Chapter 19, the immune profile elicited by mucosal ISS-ODN based vaccination appears particularly well-suited for protection against sexually transmitted diseases, such as HIV infection.

We have also evaluated the effectiveness of ISS-ODN as a mucosal and systemic adjuvant for polysaccharide antigens. In unpublished studies, we found that ISS-ODN was only a weak adjuvant for polysaccharides derived from several serotypes of *Pneumococcus*. Although antibody titers were 2–3-fold higher after immunization with pneumococcal vaccine plus ISS-ODN vs *Pneumococcal* vaccine alone, immunization with ISS-ODN did not elicit pneumococcal specific T-cell immunity. The reasons why ISS-ODN

is a potent adjuvant for protein, but not polysaccharide antigens, await further investigation. Such studies could also provide important insights into the divergent mechanisms by which immunity to protein and polysaccharide antigens develop.

3. DISCUSSION

In summary, studies from several laboratories, including our own, have established that ISS-ODN is a potent mucosal adjuvant for a wide-range of protein antigens and is effective in several strains of mice (5–9). Intranasal delivery of antigen with either ISS-ODN or CT leads to equivalent and vigorous mucosal IgA responses, whereas i.d. vaccination with ISS-ODN does not (5). However, antigen/ISS-ODN vaccination by the i.d. and i.n. routes induce equivalent systemic Th₁ biased immune responses. In contrast, i.n. antigen/CT immunization leads to secretory IgA synthesis in conjunction with a Th₂ biased systemic immune response. Additional studies establish that the adjuvant effect of ISS-ODN persists for approx seven d after i.n. delivery, and that ISS-ODN can also function as an adjuvant for oral and rectal vaccination (8,22).

The observation that equivalent mucosal IgA levels can develop in the context of Th₁ biased and Th₂ biased serum antibody responses, is consistent with other published results. Marinaro and colleagues recently demonstrated that oral delivery of tetanus toxoid with CT led to mucosal IgA production in conjunction with a Th₂ biased antibody profile. However, when tetanus toxoid and CT were coadministered with oral IL-12, the systemic antibody response was skewed toward a Th₁ phenotype, whereas mucosal IgA production was unaffected (27). Moreover, IL-12 has also been shown to be an effective mucosal adjuvant, whereas several natural infections are associated with an IgA response in conjunction with other immune parameters characteristic of Th1 biased immunity (28–30). Taken together, these observations establish that synthesis of mucosal IgA can occur in the context of both Th₁ and Th₂ biased immune responses. In addition, although several studies have suggested that IL-6 and IL-10 are produced principally by Th₂ cells, the present results confirm that IL-6 and IL-10 can be produced by both Th₁ or Th₂ cells (13,31–33).

Mucosal IgA and CTL responses are known to provide protection against a number of infectious agents. HIV is but one important example (1–4,24,34,35). There are a number of strategies available for the development of vaccines, which induce these immune parameters. However, none appear to be globally applicable. Live attenuated vaccines induce robust immune responses, including mucosal IgA synthesis and CTL activity. Unfortunately, difficulty

in attenuating many pathogens and the risk of iatrogenic disease have limited the use of live attenuated vaccines in clinical medicine (1,2,34,36). On the other hand, recombinant proteins from infectious agents are generally safe, but induce relatively poor immune responses, and generally none when delivered to mucosal surfaces. Cholera toxin is an extremely potent mucosal adjuvant for simple protein antigens, but is inherently toxic and induces a Th₂ biased antibody response, which can include the development of IgE and consequent allergic sensitization toward the target antigen (11,12). At present, such toxicity and other technical problems have kept many adjuvants, including CT, from becoming available for use in humans. Alum is essentially the only adjuvant in clinical use today. It is relatively weak, does not work with a number of antigens, does not induce CTL activity, and because it must be delivered systemically, does not induce mucosal immunity (36). A safe and effective mucosal adjuvant would be of great value in the development of better vaccines against mucosal pathogens.

ISS-ODN is a potent systemic and mucosal adjuvant, which is effective with a wide range of protein antigens, and generally induces a Th₁ biased immune response with CTL activity (5,10,37,38). Intranasal and i.d. administration of protein with ISS-ODN leads to vigorous and equivalent Th₁ biased systemic immune responses. Whereas only i.n. delivery induces mucosal immunity (5,7). Therefore, i.n. delivery of relevant antigens with ISS-ODN may well prove superior to i.d. delivery for the induction of protective immunity against mucosal pathogens.

The concept of ISS-ODN prepriming has some interesting implications with regards to its potential clinical applications. Given the prolonged adjuvant effect provided by ISS-ODN, it is conceivable that ISS-ODN could be given without antigen to modulate the course of ongoing infections. This has already been demonstrated in a model of leishmaniasis (39). By activation of the innate immune system with ISS-ODN, it may also be possible to prevent infections in an antigen independent manner. These considerations will require further investigation, but results to date suggest that ISS-ODN has a wide-range of potential clinical applications, including use as a systemic Th1 and mucosal adjuvant, and as an immunotherapeutic reagent for the treatment of ongoing infections

4. CONCLUSIONS

Immunostimulatory sequence oligodeoxynucleotides are easy to manufacture, stable, and without identified toxicity at immunogenic doses in mice, primates, and humans (26,40,41). Moreover, the authors and others, have shown that human and mouse immunocytes display similar immunologic responses to ISS-ODN (20,42,43). Because ISS-ODN is a potent systemic

and mucosal adjuvant for simple monomeric proteins, it may well prove to be a valuable reagent for the development of mucosal vaccines, and as an immunomodulatory therapeutic for the prevention or treatment of infections involving the respiratory, intestinal, and urinary mucosa. Ongoing and planned clinical trials should determine the safety and effectiveness of ISS-ODN for the prevention and treatment of infectious diseases in humans, within the next several years. If ISS-ODN proves to have a favorable therapeutic index, it will likely have a number of clinical applications in the prevention and treatment of infectious diseases.

REFERENCES

1. Czerkinsky, C., Anjuere, F., McGhee, J. R., et al. (1999) Mucosal immunity and tolerance: relevance to vaccine development. *Immunol. Rev.* **170**, 197–222.
2. Staats, H. and McGhee, J. R. (1996) Application of basic Principles of mucosal immunity to vaccine development, in *Mucosal Vaccines*, (Kiyono, H., Orga, P. L., and McGhee, J. R., eds.) Academic Press, San Diego. pp. 15–40.
3. Ada, G. L. and McElrath, M. J. (1997) HIV type 1 vaccine-induced cytotoxic T cell responses: potential role in vaccine efficacy. *AIDS Res. Hum. Retroviruses* **13**, 205–210.
4. Gallichan, W. S., Johnson, D. C., Graham, F. L., and Rosenthal, K. L. (1993) Mucosal immunity and protection after intranasal immunization with recombinant adenovirus expressing herpes simplex virus glycoprotein B. *J. Infect. Dis.* **168**, 622–629.
5. Horner, A. A., Ronaghy, A., Cheng, P. M., et al. (1998) Immunostimulatory DNA is a potent mucosal adjuvant. *Cell. Immunol.* **190**, 77–82.
6. Moldoveanu, Z., Love-Homan, L., Huang, W. Q., and Krieg, A. M. (1998) CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* **16**, 1216–1224.
7. McCluskie, M. J. and Davis, H. L. (1998) CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* **161**, 4463–4466.
8. McCluskie, M. J. and Davis, H. L. (2000) Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine* **19**, 413–422.
9. Horner, A. A., Datta, S., Takabayashi, K., (2001) Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. *J. Immunol.* **167**, 1584–1591.
10. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
11. Marinaro, M., Staats, H. F., Hiroi, T., et al. (1995) Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J. Immunol.* **155**, 4621–4629.

12. Snider, D. P., Marshall, J. S., Perdue, M. H., and Liang, H. (1994) Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. *J. Immunol.* **153**, 647–657.
13. Carpenter, E. A., Ruby, J., and Ramshaw, I. A. (1994) IFN-gamma, TNF, and IL-6 production by vaccinia virus immune spleen cells. An in vitro study. *J. Immunol.* **152**, 2652–2659.
14. Defrance, T., Vanbervliet, B., et al. (1992) Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J. Exp. Med.* **175**, 671–682.
15. Muraguchi, A., Hirano, T., Tang, B., et al. (1988) The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *J. Exp. Med.* **167**, 332–344.
16. Okahashi, N., Yamamoto, M., Vancott, J. L., et al. (1996) Oral immunization of interleukin-4 (IL-4) knockout mice with a recombinant Salmonella strain or cholera toxin reveals that CD4+ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses. *Infect. Immun.* **64**, 1516–1525.
17. Anitescu, M., Chace, J. H., Tuetken, R., et al. (1997) Interleukin-10 functions in vitro and in vivo to inhibit bacterial DNA-induced secretion of interleukin-12. *J. Interferon Cytokine Res.* **17**, 781–788.
18. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
19. Pisetsky, D. S. (1996) Immune activation by bacterial DNA: a new genetic code. *Immunity* **5**, 303–310.
20. Horner, A. A., Widhopf, G. F., Burger, J., (In Press) Immunostimulatory DNA mediated inhibition of IL-4 dependent IgE synthesis from human B cells. *J. Allergy Clin. Immunol.* **108**, 417–423.
21. Kobayashi, H., Horner, A.A., Martin-Orozco, E., and Raz, E. (2000) Pre-priming: a novel approach to DNA-based vaccination and immunomodulation. *Springer Semin. Immunopathol.* **22**, 85–96.
22. Kobayashi, H., Horner, A. A., Takabayashi, K., et al. (1999) Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity. *Cell. Immunol.* **198**, 69–75.
23. Smith, C. B. (1998) Influenza Viruses, in *Infectious Diseases* (Gornbach, S. L., Bartlett, J. G., and Blacklow, N. R., eds.) W. B. Saunders, Philadelphia. pp. 2120–2125.
24. Gallichan, W. S., Woolstencroft, R. N., Guarasci, T., McCluskie, M. J., Davis, H. L., and Rosenthal, K. L. (2001) Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus- 2 in the genital tract. *J. Immunol.* **166**, 3451–3457.
25. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947.

26. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* **106**, 124–134.
27. Marinaro, M., Boyaka, P. N., Finkelman, F. D., et al. (1997) Oral but not parenteral interleukin (IL)-12 redirects T helper 2 (Th2)- type responses to an oral vaccine without altering mucosal IgA responses. *J. Exp. Med.* **185**, 415–427.
28. Boyaka, P. N., Marinaro, M., Jackson, R. J., et al. (1999) IL-12 is an effective adjuvant for induction of mucosal immunity. *J. Immunol.* **162**, 122–128.
29. Yamamoto, M., Vancott, J.L., Okahashi, N., et al. (1996) The role of Th1 and Th2 cells for mucosal IgA responses. *Ann. N. Y. Acad. Sci.* **778**, 64–71.
30. VanCott, J. L., Franco, M. A., Greenberg, H. B., et al. (2000) Protective immunity to rotavirus shedding in the absence of interleukin- 6: Th1 cells and immunoglobulin A develop normally. *J. Virol.* **74**, 5250–5256.
31. Assenmacher, M., Schmitz, J., and Radbruch, A. (1994.) Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-gamma and in interleukin-4-expressing cells. *Eur. J. Immunol.* **24**, 1097–1101.
32. Coffman, R. L. and Mosmann, T. R. (1988) Isotype regulation by helper T cells and lymphokines. *Monogr. Allergy* **24**, 96–103.
33. Mosmann, T. R. and Coffman, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**, 145–173.
34. Letvin, N. L. (1998) Progress in the development of an HIV-1 vaccine. *Science* **280**, 1875–1880.
35. VanCott, T. C., Kaminski, R.W., Mascola, J. R., et al. (1998) HIV-1 neutralizing antibodies in the genital and respiratory tracts of mice intranasally immunized with oligomeric gp160. *J. Immunol.* **160**, 2000–2012.
36. Gupta, R. K. and Siber, G. R. (1995) Adjuvants for human vaccines—current status, problems and future prospects. *Vaccine* **13**, 1263–1276.
37. Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J., and Krieg, A. M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
38. Sato, Y., Roman, M., Tighe, H., et al. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
39. Zimmermann, S., Egeter, O., Hausmann, S., et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* **160**, 3627–3630.
40. Creticos, P. S., Eiden, J. J., Balcer, S. L., et al. (2000) Immunostimulatory oligodeoxynucleotides conjugated to Amb a 1: safety, skin test reactivity, and basophil histamine release [abstract]. *J. Allergy Clin. Immunol.* **105**, no. S70.

41. Creticos, P. S., Balcer, S. L., Schroeder, J.T., et al. (2001) Initial immunotherapy trial to explore the safety, tolerability, and immunogenicity of subcutaneous injection of the AMB a 1 immunostimulatory oligonucleotide conjugate (AIC) in ragweed allergic adults. *J. Allergy Clin. Immunol.* **107**, S216(A-712).
42. Liang, H., Nishioka, Y., Reich, C.F., Pisetsky, D.S., and Lipsky, P.E. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
43. Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.

Enhancement of the Immunoadjuvant Activity of Immunostimulatory DNA Sequence By Liposomal Delivery

Eli Kedar, Igal Louria-Hayon, Aviva Joseph, Zichria Zakay-Rones, Tomoko Hayashi, Kenji Takabayashi, and Yechezkel Barenholz

1. INTRODUCTION

Bacterial DNA and its synthetic immunostimulatory oligodeoxynucleotides' analogs (ISS-ODN or CpG motifs) are potent stimulators of both innate immunity and specific adaptive immune responses (1–5). Bacterial DNA and ISS-ODNs directly activate monocytes/macrophages, dendritic cells, natural killer (NK) cells and B cells (6–10), induce the production of proinflammatory cytokines (e.g., IL-6, IL-12, IFNs, TNF α) (4,6,7,11,12), and upregulate the expression of MHC I, MHC II and co-stimulatory molecules (9,13). In animal studies, ISS-ODNs exhibit strong Th1 (2,14–18) and mucosal adjuvanticity to a wide range of antigens (2,3,14,19–26) or allergens (27–30). Furthermore, pre-treatment with ISS-ODN, even without concomitant administration of the relevant antigens, can afford protection (for about 2 wk) against subsequent infection with intracellular pathogens (31–35), indicating activation of innate immunity.

The immunostimulatory activity of ISS-ODNs requires cellular uptake by endocytosis (36), following their binding to a cell receptor belonging to the Toll-like receptor family, TLR9 (37). Endosomal acidification, and digestion of the ODN, followed by interaction with specific protein kinases acidification results in rapid generation of reactive oxygen intermediates, leading to activation of MAPK and NF- κ B pathways and subsequent cytokine production (5,36,38).

In mice, doses of 50–100 µg of soluble ISS-ODN, and in many cases, two or more administrations, are required to achieve the desired immunomodulatory effects. This relatively high dose and repeated administration, in theory, may cause adverse reactions resulting from the proinflammatory cytokines induced (11,39). It is suggested that liposomes can serve as an efficient delivery system for ISS-based vaccines since liposomes can effectively entrap various drugs and biologicals, which are slowly released over an extended period of time, and since liposomes are rapidly and efficiently taken up by macrophages and dendritic cells (40–43). Our own studies and those of others, have demonstrated that *in vivo* liposomal delivery of antigens (42–44), cytokine, and other immunomodulators (42–47), and plasmids encoding various proteins (48,49), markedly improves immunogenicity, immunomodulatory activity, and transfection efficiency, respectively. Furthermore, for potentially toxic substances (e.g., TNFα) (50), delivery via liposomes can markedly reduce toxicity in animals. Thus, liposomal delivery of ISS-ODNs may prove beneficial for enhancing their immunomodulatory activity while reducing dose, number and frequency of administrations, and toxicity. The present study shows that the adjuvanticity of ISS-ODN in mice immunized against influenza viruses is appreciably augmented by liposomal delivery.

2. RESULTS

2.1. Encapsulation Efficiency of ISS-ODN

Endotoxin-free (1<ng/mg DNA) phosphorothioate ISS-ODN (5'-TGACTGTGAAACGTTCGAGATGA-3'), and the mutant (M) ODN(5'-TGACTGTGAAAGGTTAGAGATGA-3') (Trilink Biotechnologies, San Diego, CA) were encapsulated in large (mean diameter 1400±200 nm) multilamellar vesicles (MLV) composed of dimyristoyl-phosphatidylcholine and dimyristoyl-phosphatidylglycerol (DMPC:DMPG, 9:1 mole ratio), at a lipid:ODN ratio of 50:1–500:1 (w/w), using a modified dehydration-rehydration procedure (44), under aseptic conditions. To determine encapsulation efficiency, the liposomal preparation was centrifuged for 1 h at 45,000 rpm. The liposome precipitate and the supernatant (containing nonencapsulated ODN and traces of small liposomes) were subjected to a two-phase lipid extraction procedure (51), and the amounts of free and encapsulated ODN and liposomal phospholipids were assessed by organic phosphorus determination (52).

Using the following ratios (w/w) of lipid:ISS-ODN—50:1, 100:1, 300:1, and 500:1, the mean encapsulation efficiency (of 3 experiments) was 60, 75, 90, and 95%, respectively. No significant ODN leakage (≤10%) from the

liposomes was found after storage for three months at 4°C. To avoid overloading the mice with extra lipids, which can cause nonspecific immune stimulation (53), the formulation prepared at a 100:1 (w/w) lipid:ODN ratio was chosen for the vaccination experiments.

2.2. Enhancement of the Systemic Humoral Response by Liposomal ISS-ODN (Lip ISS-ODN) Coadministered Intramuscularly (im).

In the first experiment, mice were vaccinated once im with a divalent influenza subunit vaccine (0.15 µg protein of each strain), free (HN) and liposomal (Lip HN), alone or combined with free or Lip ISS-ODN (5, 12.5, 25 µg). Two additional groups were immunized with liposomes containing both the antigen and the ISS-ODN. M-ODN served as control. Sera were tested 3 and 12 wk postvaccination for hemagglutination inhibiting (HI) Abs (Table 1), and for antigen-specific IgG1 and IgG2a Abs (by ELISA, Table 2).

The HI titer, and the percent seroconversion of mice coimmunized with Lip ISS-ODN (Table 1, groups 6–8) were significantly greater than those of mice vaccinated with antigen alone (groups 1, 2), or with antigen combined with soluble ISS-ODN (groups 3–5). The HI titers obtained with Lip ISS-ODN were 2–8-fold higher than with soluble ISS-ODN. At 3 wk postvaccination, the seroconversion rate was 0–40% using soluble ISS-ODN or M-ODN and 100% with Lip ISS-ODN. The superior adjuvant activity of Lip ISS-ODN was seen at all doses, at both time points, and for the two viral strains. The response attained at the lowest dose (5 µg) was similar to that obtained with the highest dose (25 µg). Vaccination with liposomes co-encapsulating the antigen, and the ISS-ODN (groups 9, 10) produced a response similar to that achieved with a mixture of Lip ISS-ODN and the free antigen (groups 6–8), or with a mixture of Lip ISS-ODN and Lip HN (data not shown). M-ODN, free and liposomal, had no adjuvant effect (groups 11, 12).

Assessment of antigen-specific IgG1 and IgG2a (Table 2) showed a modest increment of IgG2a (and abrogation of IgG1) by soluble ISS-ODN (groups 3–5), and a dramatic increase in IgG2a by Lip ISS-ODN either mixed with free antigen (groups 6–8) or coentrapped with the antigen (groups 9, 10). The IgG2a levels produced by Lip ISS-ODN were 4–18 times greater than with soluble ISS-ODN. Interestingly, M-ODN, free and liposomal, slightly increased the IgG1 level and reduced the IgG2a level at 3 mo postvaccination (groups 11, 12).

Thus, a single vaccine dose containing even 5 µg of Lip ISS-ODN results in very high HI titers and specific IgG2a levels, with no IgG1 response, indicating a Th1 type. This enhanced response lasts for approx 3 mo. The results of this experiment were reproduced in two additional experiments (data not shown), using divalent and trivalent (adding the A/Sydney/5/97-

Table 1

The Antihemagglutinin Response (HI titer) Following Vaccination with Free/Liposomal Divalent Influenza Vaccine

Vaccine and dose ^a	HI titer (mean±SD) against: ^b			
	A/Beijing	B/Yamanashi	A/Beijing	B/Yamanashi
	Day 21	Day 21	Day 90	Day 90
1. HN 0.15 µg	20 ±14 (20%) ^c	6 ±5 (0%)	128±44 (100%)	12±18 (20%)
2. Lip HN	56 ±22 (100%)	24 ±17 (40%)	208±107 (100%)	48±30 (60%)
3. HN+ISS-ODN 5µg	28±11 (40%)	20 ±14 (20%)	160±150 (80%)	26±31 (20%)
4. HN+ISS-ODN 12.5 µg	24±17 (40%)	20 ±12 (20%)	44±36 (60%)	12±18 (20%)
5. HN+ISS-ODN 25 µg	20±20 (40%)	16 ±9(0%)	82±76 (60%)	18±13 (20%)
6. HN+Lip ISS-ODN 5 µg ^d	128±44 (100%)	80 ±0 (100%)	352±175 (100%)	68 ±11 (100%)
7. HN+Lip ISS-ODN 12.5 µg ^d	96 ±36 (100%)	72 ±18 (100%)	288±72 (100%)	96 ±61 (100%)
8. HN+Lip ISS-ODN 25 µg ^{d, e}	176 ±88 (100%)	144 ±36 (100%)	480±225 (100%)	144 ±36 (100%)
9. Lip (HN+ISS-ODN 5 µg)	160±0 (100%)	72 ±18 (100%)	448±175 (100%)	72 ±18 (100%)
10. Lip (HN+ISS-ODN 25 µg)	144±36 (100%)	128±44 (100%)	448±175 (100%)	104 ±54 (100%)
11. HN+M-ODN 25 µg	14 ±19 (20%)	8±8 (0%)	106±136 (60%)	8±11 (0%)
12. HN+Lip M-ODN 25 µg	30 ±10 (40%)	22±11 (20%)	176±88 (100%)	20 ±14 (20%)

^a Female BALB/c mice, 6–8-wk-old (5–6 per group), were vaccinated once (0.1 mL), im, using a divalent subunit influenza vaccine, composed of the viral surface proteins hemagglutinin and neuraminidase (HN), derived from the A/Beijing/262/95-like (H1N1) and B/Yamanashi/166/98-like strains, 0.15 µg of each. The HN antigens were given either in soluble form or entrapped in liposomes (Lip), alone, or admixed with free or liposomal ISS-ODN. The antigens and the ISS-ODN were encapsulated in DMPC/DMPG liposomes (*see Subheading 2.1*) using a lipid:antigen and lipid:ODN ratio (w/w) of 300:1 and 100:1, respectively. Groups 9, 10 were vaccinated with liposomes co-entrapping the HN antigens and the ISS-ODN. As control, mutated ODN (M-ODN) lacking the immunostimulatory sequence was used.

^b The antihemagglutinin response was tested on individual sera 21 and 90 d postvaccination, using the hemagglutination inhibition (HI) assay (54).

^c In parentheses, % seroconversion = % of mice achieving an HI titer ≥40.

^d Groups 6, 7, 8 vs groups 3, 4, 5, $p < 0.05$ (t -test) for HI titer, at both time points against the two viruses.

^e Group 8 vs group 12, $p \leq 0.01$ for HI titer, at both time points against the two viruses.

Table 2
Anti-HN IgG Isotypes Following Vaccination with a Divalent Influenza Vaccine Coadministered with Free/Liposomal ISS-ODN

Vaccine ^a	Mean IgG titer against:							
	A/Beijing d.21		B/Yamanashi d.21		A/Beijing d.90		B/Yamanashi d.90	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
1. HN 0.15 µg	<10	<10	20	<10	70	20	100	80
2. Lip HN	20	<10	80	<10	110	80	190	100
3. HN+ISS-ODN 5 µg	<10	70	<10	100	<10	350	30	450
4. HN+ISS-ODN 12.5 µg	<10	32	<10	80	<10	500	<10	380
5. HN+ISS-ODN 25 µg	<10	64	<10	70	<10	350	<10	450
6. HN+Lip ISS-ODN 5 µg	<10	450	<10	600	20	2500	<10	5000
7. HN+Lip ISS-ODN 12.5 µg	<10	560	<10	400	<10	2500	<10	4200
8. HN+Lip ISS-ODN 25 µg	<10	400	<10	600	<10	2400	<10	4600
9. Lip (HN+ISS-ODN 5 µg)	<10	400	<10	500	<10	2000	<10	4200
10. Lip (HN+ISS-ODN 25 µg)	<10	550	<10	800	10	1800	<10	5000
11. HN+M-ODN 25 µg	<10	<10	<10	<10	190	50	200	20
12. HN+Lip M-ODN 25 µg	<10	<10	<10	<10	300	20	300	10

^aSee details in Table 1. Antigen-specific isotypes were tested 21 and 90 d postvaccination on pooled sera from each group (starting at a 1/10 dilution) by ELISA (44). The antibody titer is expressed as the reciprocal of the highest serum dilution yielding 50% of the maximum absorbance obtained with “standard” immune serum, after subtracting the control (antigen + normal mouse serum).

like [H3N2], to the divalent preparation) vaccines. The HI titers on days 21–90 were 4–10-fold higher with Lip ISS-ODN (10 μ g) than with nonliposomal ISS-ODN.

In addition to testing the primary response, some groups of experiment 1 (Tables 1,2) were revaccinated 3 mo after the primary dose, and sera were tested seven days later (Table 3). Again, as seen in the primary response, repeated covaccination with Lip ISS-ODN produced HI titers 3.3–5-fold greater and IgG2a levels 3.3–8-fold higher than with soluble ISS-ODN. No increase in IgG1 was seen with either formulation. These results indicate that Lip ISS-ODN preserves its superior adjuvant activity also with repeated administration.

2.3. Induction of Delayed-Type Hypersensitivity (DTH) Response by Lip ISS-ODN

In addition to assessing the systemic humoral response, some groups of experiment 1 that were vaccinated twice at a 3-mo interval (Table 3) were tested for DTH response following subcutaneous (sc) injection of 10 μ g hemagglutinin (Table 4). A significant DTH response in all mice (5/5) was observed in group 4 vaccinated with liposomes encapsulating both the antigen and the ISS-ODN (5 μ g). Mice immunized with soluble antigen mixed with soluble or liposomal ISS-ODN (groups 2,3) developed a moderate response, and only 2/5 were considered positive. Vaccination with antigen alone, or with antigen coadministered with M-ODN (25 μ g), did not induce a response.

2.4. Enhancement of Mucosal Antiviral Response and Protective Immunity by Lip ISS-ODN Coadministered Intranasally(in)

A liposomal divalent vaccine (Lip HN, 0.5 μ g of each strain) was administered twice in, spaced one wk, alone and combined with free or Lip ISS-ODN (10 μ g), or free cholera toxin (CT, 1 μ g). In this case, HN and ISS-ODN were incorporated into the same liposomes. Serum, nasal wash, and lung homogenate were tested for antigen-specific IgG1, IgG2a, and IgA Abs four wk after the second vaccine dose. Protective immunity to viral challenge was assessed six wk postvaccination by determining lung virus titer.

Whereas soluble ISS-ODN had no adjuvant effect (except for a low increase in serum IgG2a against one viral strain), Lip ISS-ODN enhanced the levels of antigen-specific IgG2a and IgA in serum, lungs, and nasal wash, with a lower effect on IgG1 (Table 5). The response to the two viral strains included in the vaccine varied, however. Lip ISS-ODN and CT elicited similar levels of serum and mucosal IgG2a and IgA; the latter inducing higher levels of IgG1, as well. Thus, under the experimental conditions used, Lip ISS-ODN, but not soluble ISS-ODN, is capable of boosting both systemic and mucosal antigen-specific IgG2a and IgA Abs.

Table 3
The Secondary Immune Response of Mice Immunized with Free/Liposomal Divalent Influenza Vaccine

Vaccine ^a	HI titer (mean $\pm$ SD) against:		Mean IgG titer against:			
	A/Beijing	B/Yamanashi	A/Beijing		B/Yamanashi	
			IgG1	IgG2a	IgG1	IgG2a
HN 0.15 μ g	320 $\pm$ 0 (100%)	260 $\pm$ 150 (100%)	500	<10	1000	200
HN+ISS-ODN 25 μ g	896 $\pm$ 350 (100%)	368 $\pm$ 260 (100%)	400	1,000	600	3,000
HN+Lip ISS-ODN 25 μ g	3000 $\pm$ 1500 (100%)	1900 $\pm$ 800 (100%)	600	8,000	1,000	10,000
HN+M-ODN 25 μ g	640 $\pm$ 0 (100%)	170 $\pm$ 200 (50%)	500	<10	800	100

^a Mice (5–6 $\times$ group) were vaccinated 2X on d 0 and 90, and sera were tested 7 d after the second vaccine dose.

See Tables 1 and 2 for details.

Table 4
DTH Response Following Vaccination with a Divalent Influenza Vaccine Coadministered with Free/Liposomal ISS-ODN

Vaccine ^a	Increase in footpad thickness (mm) ^{b,c} Mean ±SD	Incidence of mice with DTH ^d
1. Lip HN	0.06±0.01	0/3
2. HN+ISS-ODN 5 µg	0.12±0.08	2/5
3. HN+Lip ISS-ODN 5 µg	0.16±0.09	2/5
4. Lip (HN+ISS-ODN 5 µg)	0.52±0.2	5/5
5. HN+M-ODN 25 µg	0.04±0.03	0/3

^aFor details on vaccination, see Table 1. Three mo after the first vaccine dose, mice were revaccinated. Seven d after the second vaccine dose, 10 µg of A/Beijing hemagglutinin was injected sc into the hind footpad.

^bFootpad thickness was measured prior to and 24 h after hemagglutinin injection. As control, the contralateral foot was injected with PBS (increase in footpad thickness at 24 h for PBS was 0.037 ± 0.05 mm).

^c*P* values: group 4 vs all groups ≤ 0.004; group 2 vs group 3 > 0.05 (*t*-test).

^dA > 10% increase in footpad thickness 24 h after hemagglutinin injection, relative to time 0, was considered a positive DTH response.

The remaining mice of this experiment (5 per group) were challenged in with a recombinant A/Beijing virus, and lung virus titer was quantified on d 4. As compared with unimmunized mice, the lung virus titer of mice vaccinated with Lip HN alone, Lip HN+free ISS-ODN, and Lip (HN+ISS-ODN) was reduced by 1, 2, and 3.5 logs, respectively, and 3 logs with CT (Table 6). The 30-fold difference in lung virus titer between free and liposomal ISS-ODN is in good agreement with the differences seen in HI, IgG2a, and IgA titers between these groups.

Finally, no toxicity (no change in body weight, breathing, mobility, or fur) was apparent in mice immunized with Lip ISS-ODN once or twice in and im. No swelling or ulceration was noticed at the im injection site.

3. Discussion

In this report, the authors demonstrate that ISS-ODN can be encapsulated in large multilamellar liposomes with high efficiency (up to 95%), and that the liposomal formulation is a considerably more potent parenteral and mucosal adjuvant in mice than the soluble form of ISS-ODN. A single dose of 5 µg Lip ISS-ODN was by far more potent (4–11 times, Tables 1,2) for the two viral strains than 25 µg of free ISS-ODN. The enhanced potency was reflected in both humoral and cellular responses. As with free ISS-ODN,

Table 5

Serum and Mucosal Anti-HN IgG1, IgG2a and IgA Antibodies 4 wk Following Intranasal Vaccination with a Liposomal Divalent Influenza Vaccine coadministered with Free/Liposomal ISS-ODN

Vaccine ^a (n = 5/group)	Location	Mean titer against: A/Beijing ^b			B/Yamanashi ^b		
		IgG1	IgG2a	IgA	IgG1	IgG2a	IgA
Lip HN	Serum	<20	<20	<20	<20	<20	<20
Lip HN+free ISS-ODN		<20	<20	<20	<20	40	<20
Lip (HN+ISS-ODN)		45	65	<20	100	300	90
Lip HN+CT		30	75	<20	300	100	50
Lip HN	Nasal wash	<20	<20	<20	<20	<20	<20
Lip HN+free ISS-ODN		<20	<20	<20	<20	<20	<20
Lip (HN+ISS-ODN)		90	55	110	90	60	60
Lip HN+CT		275	20	<20	150	50	50
Lip HN	Lung homogenate	<20	<20	<20	<20	<20	25
Lip HN+free ISS-ODN		<20	<20	<20	<20	<20	<20
Lip (HN+ISS-ODN)		<20	70	200	<20	20	60
Lip HN+CT		475	55	200	350	75	20

^aTwo doses (10 µL/nostril/dose) were given one week apart, using 0.5 µg antigen/dose of each viral strain. ISS-ODN (10 µg/dose) and cholera toxin (CT, 1 µg/dose) were used as adjuvants. In the combined liposomal vaccine the antigen and the ISS-ODN were co-entrapped in the same liposomes.

^bTested by ELISA (44) on pooled samples. Values obtained with normal mouse serum were subtracted. Lungs were washed 3× in cold PBS, then homogenized in 1.5 mL PBS per lungs of each mouse (referred to as 1/10 dilution). The homogenates were centrifuged at 3000 rpm for 30 min at 4°C, the supernatants were collected and centrifuged at 14,000 rpm for 20 min at 4°C, and antibody titer in the supernatants was measured. Nasal wash was made in 0.2 mL PBS (referred to as 1/10). Serial two-fold dilutions were tested, starting with 1/20 sample dilution.

Table 6
Protection Against Influenza Virus Infection 6 wk Following Intranasal Vaccination with a Divalent Influenza Vaccine Coadministered with Free/Liposomal ISS-ODN

Vaccine (n = 5) ^a	Lung virus titer (log10) ^b
Unimmunized	6
Lip HN	5
Lip HN + free ISS-ODN	4
Lip (HN+ISS-ODN)	2.5
Lip HN + CT	3

^aSee Table 5 for details.

^bSix weeks after the second vaccine dose, mice were anesthetized and 25 μ L of live virus suspension per nostril was administered, using the reassortant virus X-127 (A/Beijing/262/95 (H1N1) $\times$ X-31 (A/Hong Kong/1/68 $\times$ A/PR/8/34), which is infectious to mice, 10⁷ EID 50 (egg-infectious dose 50%). The lungs were removed on day 4, washed 3 $\times$ in cold PBS, and homogenized in 1.5 mL PBS per lungs/mouse (referred to as 1/10 dilution). Homogenates of each group were pooled and centrifuged at 2000 rpm for 30 min at 4°C and the supernatants collected. Serial 10-fold dilutions were performed and 0.2 mL of each dilution was injected in duplicate into the allantoic sac of embryonated chicken eggs. After 48 h at 37°C, 0.1 mL of allantoic fluid was removed and checked for viral presence by hemagglutination with chicken erythrocytes. The lung virus titer is determined as the highest dilution of lung homogenate producing virus in the allantoic fluid (positive hemagglutination). A titer of .5 indicates 1 egg was positive and 1 egg was negative at the highest dilution.

Lip ISS-ODN mainly enhanced Th1-biased immunity, based on the IgG1 and IgG2a levels, particularly upon im administration.

The ability of Lip-ISS-ODN to enhance a Th1-dominant response was further substantiated by recent preliminary experiments (data not shown), in which splenocytes obtained 6 wk after a single im dose of an influenza vaccine, coadministered with free or liposomal ISS-ODN (10 μ g), were stimulated in vitro with the antigen for 3–6 d. The proliferative response, CTL response against virus-infected cells, and IFN γ production were 3–5 times greater using Lip-ISS-ODN as an adjuvant, as compared with free ISS-ODN.

Previously, we (44,45,50,53) and others (42,46,47) reported that liposomal delivery of various cytokines potentiates their bioactivity in various experimental models. The sustained release of the liposome-associated agent (depot effect) at the injection site (41–43); the expression of a large portion of the entrapped molecules on the outer liposome membrane (as found with cytokines) (45); the efficient uptake of liposomes by antigen-presenting

cells, leading to antigen processing via both MHC I and MHC II pathways (43); and the possibility of activating intracellular receptors can be accounted for the superior adjuvant action of Lip ISS-ODN.

Several studies showed enhanced adjuvant activity of ISS-ODN by chemical coupling to the antigen (20,28,29,55) and by mixing alum-adsorbed antigen with ISS-ODN (56,57). In contrast to the Th2 profile obtained for alum-based immunizations (56), our findings suggest that liposomal delivery of ISS maintains the Th1 profile of ISS-based vaccines.

In the experiments, the same increment in the humoral response was obtained when free or liposomal antigen was mixed with Lip ISS-ODN (in separate liposomes), and when the antigen and the ISS-ODN were co-entrapped in the same liposomes. Our results differ from those reported recently by two groups, on liposomal plasmid DNA and liposomal ISS-ODN. Gürsel et al. (58) found that the adjuvant activity of a noncoding plasmid was demonstrated only when the plasmid and the hepatitis B surface antigen were co-entrapped within the same liposomes, but not in separate liposomes. Ludewig et al. (59) showed that the antiviral and antitumor responses following vaccination with liposome-entrapped peptides could be augmented by ISS-ODN. However, the response obtained with liposomal antigens admixed with nonliposomal ISS-ODN was similar to that produced by antigens and ISS-ODN incorporated into the same liposomes. In the present study, ISS-ODN was an equally effective adjuvant (for the humoral response) when entrapped in the same liposomes with the antigen or in separate liposomes. Moreover, Lip ISS-ODN was always more effective than free ISS-ODN (5–25 µg). These differences may result from:

1. The different and more efficient procedure used by us for the encapsulation of ISS-ODN, and the different chemical composition and size of the liposomes, both of which may affect the localization of the ISS-ODN in the liposome and its delivery.
2. Differences in the experimental systems, including the antigens and the type of ISS-ODNs, the route of administration, the dosage, and the assays used to monitor the response.

It should be noted, however, that in contrast to the effect of Lip ISS-ODN on the systemic humoral response, the DTH response (Table 4) was greater in our study when the antigen and the ISS-ODN were coencapsulated, as compared with the DTH response obtained with liposomal antigen mixed with liposomal ISS-ODN (in separate liposomes). In our forthcoming experiments, varying ISS-ODN doses and additional parameters of cellular response (e.g., cytokine production, proliferative response, CTL activity) will be tested to determine whether boosting of humoral and cellular

responses requires the same, or a different mode of ISS-ODN delivery by liposomes.

Using in immunization, we observed a similar adjuvant activity for Lip ISS-ODN and soluble CT, a "classical" mucosal adjuvant (60), as determined by the IgG2a and IgA levels and protective immunity. In addition, CT increased more effectively the mucosal IgG1 than the IgG2a response. The induction of systemic and mucosal IgA and IgG1 (both isotypes are Th2 directed) following in (Table 5), but not im (Table 2), coadministration of Lip ISS-ODN suggests that the Th1-Th2 dichotomy with regard to mucosal immunization is not as strict as that seen following parenteral immunization. The elicitation of high levels of mucosal antigen-specific IgA, following nonparenteral covaccination with soluble ISS-ODN also was reported by others (14,23–25,61).

In light of the similar adjuvant potency of Lip ISS-ODN and CT, and the lack of apparent toxicity of the latter, Lip ISS-ODN appears to be a safe and efficient parenteral and mucosal vaccine adjuvant, easy to prepare and store, and with wide applications, such as for infectious diseases, cancer, and allergy.

ACKNOWLEDGMENTS

Supported by research grants from the Israel Science Foundation and the Y. Horowitz Foundation, and NIH grant AI40682 (KT). We are indebted to Drs. R. Glück and R. Zurbriggen, Berna Bioech, Bern, Switzerland, for providing the influenza subunit vaccines. We would also like to thank Dr. Eyal Raz, University of California, San Diego, for his assistance and advice.

REFERENCES

1. Raz, E. (ed.) (2000) Immunostimulatory DNA sequences (ISS). *Springer Semin. Immunopathol.* **22**, 1–183.
2. Krieg, A. M. (2000) Immune effects and mechanisms of action of CpG motifs. *Vaccine* **19**, 618–622.
3. Davis, H.L. (2000) Use of CpG DNA for enhancing specific immune responses. *Curr. Top. Microbiol. Immunol.* **247**, 171–183.
4. Van Uden, J. and Raz, E. (2000) Introduction to immunostimulatory DNA sequences. *Springer Semin. Immunopathol.* **22**, 1–9.
5. Pisetsky, D. S. (2000) Mechanisms of immune stimulation by bacterial DNA. *Springer Semin. Immunopathol.* **22**, 21–33.
6. Halpern, M. D., Kurlander, R. J., and Pisetsky, D. S. (1996) Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell. Immunol.* **167**, 72–78.
7. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to

- secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
8. Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
 9. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
 10. Yamamoto, S., Yamamoto, T., Iho, S., and Tokunga, T. (2000) Activation of NK cell (human and mouse) by immunostimulatory DNA sequence. *Springer Semin. Immunopathol.* **22**, 35–43.
 11. Lipford, G. B., Sparwasser, T., Bauer, M., et al. (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* **27**, 3420–3426.
 12. Klinman, D. M., Kamstrup, S., Verthelyi, D., et al. (2000) Activation of the innate immune system by CpG oligodeoxynucleotides: immunoprotective activity and safety. *Springer Semin. Immunopathol.* **22**, 173–183.
 13. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M.-D., Kornbluth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
 14. Horner, A. A., Cinman, N., Ronaghy, A., and Raz, E. (2000) Mucosal adjuvanticity of immunostimulatory DNA sequences. *Springer Semin. Immunopathol.* **22**, 133–146.
 15. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
 16. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V., and Harding, C. V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* **186**, 1623–1631.
 17. Heeg, K. and Zimmermann, S. (2000) CpG DNA as a Th1 trigger. *Int. Arch. Allergy Immunol.* **121**, 87–97.
 18. McCluskie, M.J. and Davis, H.L. (1999) CpG DNA as mucosal adjuvant. *Vaccine* **18**, 231–237.
 19. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigens: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344.
 20. Klinman, D. M., Barnhart, K. M., and Conover, J. (1999) CpG motifs as immune adjuvants. *Vaccine* **17**, 19–25.
 21. Davila, E. and Celis, E. (2000) Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. *J. Immunol.* **165**, 539–547.
 22. Cho, H. J., Takabayashi, K., Cheng, P. M., Nguyen, M. D., Corr, M., Tuck, S., and Raz, E. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic

- lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514.
23. Horner, A. A. and Raz, E. (2000) Immunostimulatory sequence oligodeoxynucleotide: A novel mucosal adjuvant. *Clin. Immunol.* **95**, S19–29.
 24. McCluskie, M. J., Weeratna, R. D., and Davis, H. L. (2001) The potential of oligodeoxynucleotides as mucosal and parenteral adjuvants. *Vaccine* **19**, 2657–2660.
 25. McCluskie, M. J. and Davis, H. L. (2001) Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine* **19**, 413–422.
 26. Krieg, A. M. and Davis, H. L. (2001) Enhancing vaccines with immune stimulatory CpG DNA. *Curr. Opin. Mol. Ther.* **3**, 15–24.
 27. Askenase, P. W. (2000) Gee whiz: CpG DNA allergy therapy! *J. Allergy Clin. Immunol.* **106**, 124–134.
 28. Shirota, H., Sano, K., Kikuchi, T., Tamura, G., and Shirato, K. (2000) Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J. Immunol.* **164**, 5575–5582.
 29. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen amb a1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* **106**, 124–134.
 30. Horner, A. A., Van Uden, J. H., Zubeldia, J. M., Broide, D., and Raz, E. (2001) DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol. Rev.* **179**, 102–118.
 31. Krieg, A. M., Love-Homan, L., Yi, A. K., and Harty, J. T. (1998) CpG DNA induces sustained IL-12 expression *in vivo* and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* **161**, 2428–2434.
 32. Elkins, K. L., Reinehart-Jones, T. R., Stibitz, S., Conover, J. S., and Klinman, D. M. (1999) Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* **162**, 2291–2298.
 33. Wagner, H., Häcker, H., and Lipford, G.B. (2000) Immunostimulatory DNA sequences help to eradicate intracellular pathogens. *Springer Semin. Immunopathol.* **22**, 147–152.
 34. Kobayashi, H., Horner, A. A., Martin-Orozco, E., and Raz, E. (2000) Pre-priming: a novel approach to DNA-based vaccination and immunomodulation. *Springer Semin. Immunopathol.* **22**, 85–96.
 35. Gramzinski, R. A., Doolan, D. L., Sedegah, M., Davis, H. L., Krieg, A. M., and Hoffman, S. L. (2001) Interleukin-12- and gamma interferon-dependent protection against malaria conferred by CpG oligodeoxynucleotide in mice. *Infect. Immun.* **69**, 1643–1649.
 36. Krieg A. M. (2000) Signal transduction induced by immunostimulatory CpG DNA. *Springer Semin. Immunopathol.* **22**, 97–105.
 37. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.

38. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
39. Wagner, H., Lipford, G. B., and Häcker, H. (2000) The role of immunostimulatory CpG-DNA in septic shock. *Springer Semin. Immunopathol.* **22**, 167–171.
40. Barenholz, Y. and Crommelin, D. J. A. (1994) Liposomes as pharmaceutical dosage forms, in *Encyclopedia of pharmaceutical technology*, vol. 9. (Swarbrick, J. and Boylan, E. C., eds.), Marcel Dekker, New York, pp. 1–39.
41. Kersten, G. F. and Crommelin, D. J. A. (1995) Liposomes and ISCOMS as vaccine formulations. *Biochim. Biophys. Acta* **1241**, 117–138.
42. Gregoriadis, G., Gursel, I., Gursel, M., and McCormack, B. (1996) Liposomes as immunological adjuvants and vaccine carriers. *J. Controlled Release* **41**, 49–56.
43. Alving, C. R. (1997) Liposomes as adjuvants for vaccines, in *New generation vaccines*, 2nd ed. (Levine, M. M., Woodrow, G. C., Kaper, J. B., and Cobon, G. S., eds.), Marcel Dekker, New York, pp. 207–213.
44. Babai, I., Samira, S., Barenholz, Y., Zakay-Rones, Z., and Kedar, E. (1999) A novel influenza subunit vaccine composed of liposome-encapsulated haemagglutinin/neuraminidase and IL-2 or GM-CSF. II. Induction of TH1 and TH2 responses in mice. *Vaccine* **17**, 1239–1250.
45. Kedar, E. and Barenholz, Y. (1998) Delivery of cytokines by liposomes: a means of improving their immunomodulatory and antitumor activity, in *The biotherapy of cancers: from immunotherapy to gene therapy* (Chouaib S., ed.), INSERM, Paris, pp. 333–362.
46. Anderson, P. M., Hanson, D. C., Hasz, D. E., Halet, M. R., Blazar, B. R., and Ochoa, A. C. (1994) Cytokines in liposomes: preliminary studies with IL-1, IL-2, IL-6, GM-CSF and interferon- γ . *Cytokine* **6**, 92–101.
47. Lachman, L. B., Shih, L.-C. N., Rao, X.-M., et al. (1995) Cytokine-containing liposomes as adjuvants for HIV subunit vaccines. *AIDS Res. Hum. Retroviruses* **11**, 921–932.
48. Gregoriadis, G., Saffie, R., and de Souza, J.B. (1997) Liposome mediated DNA vaccination *FEBS Lett.* **401**, 107–110.
49. Gurunathan, S., Klinman, D. M., and Seder, R. A. (2000) DNA vaccines: immunology, application, and optimization. *Annu. Rev. Immunol.* **18**, 927–974.
50. Kedar, E., Palgi, O., Golod, G., Babai, I., and Barenholz, Y. (1997) Delivery of cytokines by liposomes. III. Liposome-encapsulated GM-CSF and TNF- α show improved pharmacokinetics and biological activity and reduced toxicity in mice. *J. Immunother.* **20**, 180–193.
51. Bligh, E. J. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
52. Barenholz, Y. and Amselem, S. (1993) Quality control assays in the development and clinical use of liposome-based formulations, in *Liposome technology*, 2nd ed., vol I. (Gregoriadis G, ed.), CRC Press, Boca Raton, FL, pp. 527–616.
53. Kedar, E., Gur, H., Babai, I., Samira, S., Even-Chen, S., and Barenholz, Y. (2000) Delivery of cytokines by liposomes: Hematopoietic and immunomodulatory activity of interleukin-2 encapsulated in conventional liposomes and in long-circulating liposomes. *J. Immunother.* **23**, 131–145.

54. Sever, J. (1962) Application of microtechnique to viral serologic investigation. *J. Immunol.* **88**, 321–325.
55. Tighe, H., Takabayashi, K., Schwartz, D., Marsden, R., Beck, L., Corbeil, J., et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947.
56. Weeratna, R. D., McCluskie, M. J., Xu, Y., and Davis, H. L. (2000) CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* **18**, 1755–1762.
57. Eastcott, J. W., Holmberg, C. J., Dewhirst, F. E., Esch, T. R., Smith, D. J., and Taubman, M. A. (2001) Oligonucleotide containing CpG motifs enhances immune responses to mucosally or systemically administered tetanus toxoid. *Vaccine* **19**, 1636–1642.
58. Gürsel, M., Tunca, S., Özkan, M., Özcengiz, G., and Alaeddinoglu, G. (1999) Immunoadjuvant action of plasmid DNA in liposomes. *Vaccine*. **17**, 1376–1383.
59. Ludewig, B., Barchiesi, F., Pericin, M., Zinkernagel, R. M., Hengartner, H., and Schwendener, R. A. (2001) *In vivo* antigen loading and activation of dendritic cells via a liposomal peptide vaccine mediates protective antiviral and antitumor immunity. *Vaccine* **19**, 23–32.
60. Snider, D. P. (1995) The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. *Crit. Rev. Immunol.* **15**, 317–348.
61. Gallichan, W. S., Woolstencroft, R. N., Guarasci, T., McCluskie, M. J., Davis, H. L., and Rosenthal, K. L. (2001) Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. *J. Immunol.* **166**, 3451–3457.

Immunostimulatory Sequences in Plasmid Vectors

**Christina C. N. Wu, Chih Min Tang,
Brian Crain and Maripat Corr**

1. INTRODUCTION

Immunization with plasmid DNA (pDNA) results in potent humoral and cellular immune responses that are specific to the expressed antigen. Intradermal injection of plasmid DNA (pDNA) in normal saline induces T helper cell differentiation to the T helper 1 type (Th1). This response is characterized by high levels of interferon (IFN γ) and the production of Isotype G2a antibodies, as well as the activation of strong major histocompatibility complex (MHC) class I-restricted cytotoxic T-lymphocyte responses (1–5). Additionally, immunization with plasmid DNA can dictate the dominant phenotype of the overall response. Not only can an antigen-specific Th2 response be switched to an antigen-specific Th1 response by plasmid DNA immunization, but the Th1 response induced by pDNA immunization also prevails over a later attempt to induce a Th2 response by protein in alum immunization (2). This bias in the T-cell cytokine profile and the potent response relative to the quantitatively small amount of protein produced by the pDNA injection suggested that the DNA was acting as an adjuvant to enhance the response.

The elements in plasmid DNA that augment the immune response were initially localized to the noncoding region in the backbone of the plasmid vector and are referred to as immunostimulatory sequences (ISS). ISS are typically palindromic hexamer motifs containing central cytosine-guanine (CpG) dinucleotides. The immunogenicity of pDNA injection is significantly reduced by cytosine methylation and increased by coadministering exogenous CpG-containing DNA (6). Also, altering the number of ISS within the plasmid can influence the magnitude of the Th1 response (7).

These unmethylated CpG sequences have antigen independent effects on the immune system, which may then lead to more robust responses of the adaptive immune system. The pleiotropic roles of ISS in plasmid DNA are discussed further in **Subheading 2**.

2. HISTORICAL PERSPECTIVE

The innate immunostimulatory properties of DNA were originally observed in experiments attempting to define the antitumor activity of an extract of *Mycobacterium bovis* (8,9). A nucleic acid-rich fraction extracted and purified from bacillus Calmette-Guérin (MY-1) augmented natural killer (NK) cell activity of mouse spleen cells in vitro, induced IFN-alpha/beta and -gamma production, and rendered normal macrophages cytotoxic toward tumor cells (10). These cellular responses were induced by the MY-1 digested preliminarily with RNase, but not by the MY-1 digested with DNase, indicating that DNA contained in MY-1 was essential for the responses (11). The stimulation of NK cell activity could be reproduced with small synthetic oligonucleotides (30 basepairs or less) (12,13). The characteristics of stimulatory oligonucleotides were further defined as those having a hexamer palindromic motif and a central CpG dinucleotide (14). These original observations with microbial DNA would later be extended to other unmethylated forms of DNA containing the CpG motifs.

3. ISS IN pDNA

The seminal finding of Wolf and colleagues that plasmid DNA injected into the muscle of mice could result in the expression of the encoded protein and generate an immune response opened the field to a wide range of investigations studying these phenomena (15). Initially there was a seeming incongruity between the low levels of protein expressed by the injected plasmid and the magnitude of the elicited immune response. The augmentation of the immune response was localized to specific sequences (ISS) in the noncoding region of the plasmid vector or in the antibiotic resistance gene (7).

These ISS were similar to those described in mycobacterial DNA, namely palindromic hexamer motifs containing central CpG dinucleotides (7). Unmethylated microbial DNA triggers a response from the innate branch of immunity in a mammalian host, characterized by the production of IL-6, IL-12, and IFN- γ . In vitro experiments showed that plasmid DNA induced production of the same cytokines that were stimulated by bacterial DNA. Additionally, in vivo experiments showed that the immunogenicity of a DNA vaccine was significantly reduced by methylating its CpG motifs and was significantly increased by coadministering exogenous CpG-containing DNA (6).

The role of these sequences as an inherent adjuvant is further supported by experiments showing that the coinjection of protein and ISS containing oligonucleotides (16), or antigen combined with incomplete Freund's adjuvant and ISS containing oligonucleotides (17), can elicit antigen-specific Th1 responses. Furthermore, ISS containing oligonucleotides covalently linked to antigen are more potent than gene or protein vaccination alone, or immunization with mixtures of antigen and ISS containing oligonucleotides in raising Th1 cellular and associated antibody response (18,19).

At this point there have been literally hundreds of deoxyoligonucleotide sequences tested for their influences on the immune system. Generally, an ISS consensus motif of 5'-purine-purine-C-G-pyrimidine-pyrimidine-3' is often cited (20). This motif has a strong stimulatory influence on murine cells, but may not be as potent with human cells (21). The optimal motif for human cells has been proposed to be 5'-purine-TCG-pyrimidine-pyrimidine-3' (21). These stimulatory sequence modules are not definitive, as there are sequences without a CpG motif that are still stimulatory (22,23). Also, subsets of oligonucleotides are being further defined. For example, two distinct clusters of synthetic oligonucleotides were found to preferentially stimulate human peripheral B cells and NK cells (24). The exact length of ISS containing sequences has also been the subject of intensive study. Phosphorothioate-based ISS-oligonucleotides as short as eight bases have been demonstrated to retain biologic activity (20). In contrast other reports indicate that although activity is seen with oligonucleotides that are 15–18 bases in length slightly longer oligonucleotides of 22 bases may be more potent (25,26).

Although additional ISS sequences may augment the adjuvant effect (7), the stimulatory effects of multiple ISS incorporated into plasmid vectors are not directly additive. There is a limit to the value of adding additional ISS sequences to plasmid vectors (16). Also there are sequences that are similarly felt to be immunoinhibitory. Despite comparable levels of unmethylated CpG dinucleotides, DNA from serotype 12 adenovirus was immunostimulatory, but serotype 2 was not. It was noted that in type 2 adenoviral DNA the ISS motifs are outnumbered by a 15- to 30-fold excess of CpG dinucleotides in clusters of direct repeats or with a C on the 5' side or a G on the 3' side. Synthetic oligodeoxynucleotides containing these putative neutralizing (CpG-N) motifs blocked immune activation by ISS motifs both *in vitro* and *in vivo*. Eliminating 52 of the 134 CpG-N motifs present in a DNA vaccine markedly enhanced its Th1-like function *in vivo*, which was increased further by the addition of ISS motifs (27). The complexities that are unfolding suggest that these motifs may not result in a simple summation of their expected activity.

4. INFLUENCES ON T-CELL PRIMING

Multiple mechanisms contribute to the induction of Th1 responses by DNA immunization. T cells, however, are not directly stimulated by ISS. Antigen presenting cells (APCs) such as monocytes/macrophages do make several Th1-inducing cytokines and upregulate several costimulators in response to the ISS present in pDNA (28). The ISS or CpG motifs in plasmid DNA may also enhance the ability of the APC to effectively present antigen to the T cell, or may change the overall environment of injected tissues to promote Th1 differentiation. Unmethylated DNA sequences are able to elicit a release of cytokines such as type 1 IFN from cells of the innate immune system (29). Freshly isolated human monocytes increase expression of IFN α , IFN β , IL-12, and IL-18 mRNA *in vitro* upon transfection with ISS (16). These cytokines induce IFN γ , which promotes differentiation of naive Th0 cells to Th1 (30–33). Other cytokines produced with exposure to ISS include IL-6, IL1 β , IL-1RA, MIP-1 β , and MCP-1 (11,16,29,34–36). Hence, the local environment may be preconditioned to confer a Th1 bias to the immune response to the plasmid encoded antigen.

The ISS in plasmid DNA effects not only soluble factors, but also results in enhanced expression of surface ligands on antigen presenting cells (37). This upregulation of costimulatory molecules can be directly attributed to ISS motifs (28). Presumably the presence of a greater number of costimulatory ligands on APC enhances their antigen presentation function. This expression however is insufficient to replace the need for MHC class II-restricted CD4⁺ T cell help in the priming of a CTL response. MHC class II deficient mice are unable to generate a CTL response following pDNA immunization (38). This deficit in CTL priming in MHC class II-deficient mice is only modestly restored with CD40-activating antibody, suggesting that other factors are provided by MHC class II-restricted T-cell help for CTL induction. These additional elements cannot be substituted with the response to ISS in pDNA.

The effects that plasmid DNA has on the APC may derive from intra- and extracellular mechanisms. Dendritic cells are present in the skin or can become recruited to the site of injection in other tissues. Directly transfected dendritic cells following both needle injection (37,39) as well as biolistic immunization (40,41) have been described. In these reports, cells with dendritic cell surface markers or morphology have been demonstrated to contain plasmid DNA sequences, to express the plasmid encoded antigen, or to elicit a cytokine response from an antigen-specific T-cell hybridoma.

Some of the adjuvant effects of plasmid DNA may be mediated by extracellular interaction with a Toll-like receptor (TLR9). TLR9-deficient

(TLR9^{-/-}) mice did not show any response to CpG DNA, including proliferation of splenocytes, inflammatory cytokine production from macrophages, and maturation of dendritic cells (42). This receptor ligand recognition was further shown to be species specific. The optimal CpG motif for hTLR9 was 5'-GTCGTT-3', whereas, the optimal murine sequence was 5'-GACGTT-3' (42). The recognition of CpG by a Toll-like receptor family member suggests that this signaling pathway is linked to the strong influence that ISS have on the innate immune system. Future experiments using TLR9 deficient mice should elucidate the role of this signaling pathway in generating the immune response to plasmid DNA injection.

There may be other pathways involved however, as the cellular uptake of ISS may be necessary for murine B-cell activation (20). ISS containing oligodeoxynucleotides and bacterial DNA activate DNA-PK, which in turn contributed to activation of IKK and NF- κ B (43,44). In vitro stimulation of bone marrow derived macrophages from mice lacking the catalytic subunit of DNA-PK (DNA-PKcs) with ISS containing oligonucleotides resulted in defective induction of IL-6 and IL-12 (44). If there is a separate molecular pathway that is activated by the presence of intracellular unmethylated DNA then specific sequences could be selected to utilize this pathway advantageously.

5. CONCLUSION

Intensive research has been performed in the search for the optimal combination of inhibitory and stimulatory DNA motifs. These sequences could prove to be important in optimizing vaccine design. The recent identification of a Toll like receptor family member as a specific receptor for CpG DNA will likely galvanize further efforts toward engineering the perfect delivery vector.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (AI40682, AR40770, AR44850). We are grateful to Noon, N. and Uhle, J. for secretarial assistance.

REFERENCES

1. Manickan, E., Rouse, R. J., Yu, Z., Wire, W. S. and Rouse, B. T. (1995) Genetic immunization against herpes simplex virus. Protection is mediated by CD4⁺ T lymphocytes, *J. Immunol.* **155**, 259–265.
2. Raz, E., Tighe, H., Sato, Y., et al. (1996) Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA*, **93**, 5141–5145.

3. Feltquate, D. M., Heaney, S., Webster, R. G. and Robinson, H. L. (1997) Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* **158**, 2278–2284.
4. Ulmer, J. B., Donnelly, J. J., Parker, S. E., et al. (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein, *Science* **259**, 1745–1749.
5. Davis, H. L., Schirmbeck, R., Reimann, J. and Whalen, R. G. (1995) DNA-mediated immunization in mice induces a potent MHC class I-restricted cytotoxic T lymphocyte response to the hepatitis B envelope protein. *Hum. Gene Ther.* **6**, 1447–1456.
6. Klinman, D. M., Yamshchikov, G. and Ishigatsubo, Y. (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* **158**, 3635–3639.
7. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
8. Shimada, S., Yano, O., Inoue, H., et al. (1985) Antitumor activity of the DNA fraction from *Mycobacterium bovis* BCG. II. Effects on various syngeneic mouse tumors. *J. Natl. Cancer Inst.* **74**, 681–688.
9. Tokunaga, T., Yamamoto, H., Shimada, S., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–962.
10. Shimada, S., Yano, O., and Tokunaga, T. (1986) In vivo augmentation of natural killer cell activity with a deoxyribonucleic acid fraction of BCG. *Jpn. J. Cancer Res.* **77**, 808–816.
11. Yamamoto, S., Kuramoto, E., Shimada, S., and Tokunaga, T. (1988) In vitro augmentation of natural killer cell activity and production of interferon-alpha/beta and -gamma with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **79**, 866–873.
12. Kuramoto, E., Yano, O., Kimura, Y., et al. (1992) Oligonucleotide sequences required for natural killer cell activation. *Jpn. J. Cancer Res.* **83**, 1128–1131.
13. Tokunaga, T., Yano, O., Kuramoto, E., et al. (1992) Synthetic oligonucleotides with particular base sequences from the cDNA encoding proteins of *Mycobacterium bovis* BCG induce interferons and activate natural killer cells. *Microbiol. Immunol.* **36**, 55–66.
14. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN [correction of INF] and augment IFN-mediated [correction of INF] natural killer activity. *J. Immunol.* **148**, 4072–4076.
15. Wolff, J. A., Malone, R. W., Williams, P., et al. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465–1468.
16. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.

17. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V., and Harding, C. V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity, *J. Exp. Med.* **186**, 1623–1631.
18. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947.
19. Cho, H. J., Takabayashi, K., Cheng, P. M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514.
20. Krieg, A. M., Yi, A. K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
21. Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S., and Lipsky, P. E. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
22. Branda, R. F., Moore, A. L., Lafayette, A. R., et al. (1996) Amplification of antibody production by phosphorothioate oligodeoxynucleotides. *J. Lab. Clin. Med.* **128**, 329–338.
23. Monteith, D. K., Henry, S. P., Howard, R. B., et al. (1997) Immune stimulation—a class effect of phosphorothioate oligodeoxynucleotides in rodents. *Anticancer Drug Des.* **12**, 421–432.
24. Verthelyi, D., Ishii, K., Gursel, M., Takeshita, F., and Klinman, D. (2001) Human peripheral blood cells differentially recognize and respond to two distinct CPG motifs. *J. Immunol.* **166**, 2372–2377.
25. Ballas, Z. K., Rasmussen, W. L., and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
26. Yamamoto, T., Yamamoto, S., Kataoka, T., and Tokunaga, T. (1994) Ability of oligonucleotides with certain palindromes to induce interferon production and augment natural killer cell activity is associated with their base length. *Antisense Res. Dev.* **4**, 119–122.
27. Krieg, A. M., Wu, T., Weeratna, R., et al. (1998) Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc. Natl. Acad. Sci. USA* **95**, 12,631–12,636.
28. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M., Kornbluth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences *Int. Immunol.* **11**, 1111–1113.
29. Sun, S., Zhang, X., Tough, D. F., and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* **188**, 2335–2342.
30. Brinkmann, V., Geiger, T., Alkan, S., and Heusser, C. H. (1993) Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. *J. Exp. Med.* **178**, 1655–1663.
31. Yaegashi, Y., Nielsen, P., Sing, A., Galanos, C., and Freudenberg, M. A. (1995) Interferon beta, a cofactor in the interferon gamma production induced by gram-negative bacteria in mice. *J. Exp. Med.* **181**, 953–960.

32. Trinchieri, G. (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* **13**, 251–276.
33. Okamura, H., Tsutsi, H., Komatsu, T., et al. (1995) Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* **378**, 88–91.
34. Yi, A. K., Chace, J. H., Cowdery, J. S., and Krieg, A. M. (1996) IFN-gamma promotes IL-6 and IgM secretion in response to CpG motifs in bacterial DNA and oligodeoxynucleotides. *J. Immunol.* **156**, 558–564.
35. Yi, A. K., Klinman, D. M., Martin, T. L., Matson, S. and Krieg, A. M. (1996) Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J. Immunol.* **157**, 5394–5402.
36. Schwartz, D. A., Quinn, T. J., Thorne, P. S., Sayeed, S., Yi, A. K., and Krieg, A. M. (1997) CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* **100**, 68–73.
37. Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. (1999) DNA vaccination: transfection and activation of dendritic cells as key events for immunity, *J. Exp. Med.* **189**, 169–718.
38. Chan, K., Lee, D. J., Schubert, A., et al. (2001) The roles of MHC class II, CD40, and B7 costimulation in CTL induction by plasmid DNA, *J. Immunol.* **166**, 3061–3066.
39. Casares, S., Inaba, K., Brumeanu, T. D., Steinman, R. M., and Bona, C. A. (1997) Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J. Exp. Med.* **186**, 1481–1486.
40. Porgador, A., Irvine, K. R., Iwasaki, A., Barber, B. H., Restifo, N. P., and Germain, R. N. (1998) Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization, *J. Exp. Med.* **188**, 1075–1082.
41. Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K. M and Falo, L. D., Jr. (1996) DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* **2**, 1122–1128.
42. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
43. Yi, A. K. and Krieg, A. M. (1998) CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel, *J. Immunol.* **160**, 1240–1245.
44. Chu, W., Gong, X., Li, Z., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.

PART V
APPLICATIONS—INFECTIOUS DISEASES

Comparison of CpG DNA with Other Adjuvants for Vaccination Against Hepatitis B

Michael J. McCluskie, Risini D. Weeratna,
and Heather L. Davis

1. INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide. More than 2 billion people have been infected with HBV, of which approx 350 million remain chronically infected, and of whom 25% will ultimately die as a result of hepatocellular carcinoma or cirrhosis (1). The first vaccines against HBV to be used in humans comprised noninfectious 22 nm hepatitis B surface antigen (HBsAg) particles, purified from the plasma of chronic carriers. Although plasma-derived vaccines were effective, they were not developed in many areas of the world for mass immunization because of long and expensive purification procedures, the limited supply of chronically infected human plasma, or perceived safety issues (2). Today, most HBV vaccines contain recombinant HBsAg, which is usually derived from yeast, and are given by intramuscular (im) injection. Current HBsAg-containing vaccines contain aluminum hydroxide (alum), the most common adjuvant used in human vaccines, and the only one licensed for use in humans in most countries. In 1991, the World Health Assembly announced the target of introducing an HBV vaccine into national immunization programs in all countries by 1997. This was not achieved, but to date more than 100 countries have added it into their immunization programs, including most industrial countries in Western Europe, Eastern Asia and the Middle East, North America, and Australia (3). However, despite the availability of effective HBV vaccines, widespread application of HBV vaccines has been restricted on account of the high cost and multiple immu-

nization schedule required (typically 0, 1 and 6 or 0, 1 and 12 mo), and extremely low immunization rates are still found in the poorest countries in the world, including many countries in sub-Saharan Africa, the Indian sub-continent, and in the Newly Independent States (3).

One strategy that has been proposed to improve the efficacy of HBV vaccines is the use of alternative adjuvants to alum. CpG DNA is a novel adjuvant that contains unmethylated CpG dinucleotides in particular base contexts (CpG motifs), and which is given most often in the form of synthetic oligodeoxynucleotides (CpG ODN) made with a nuclease-resistant phosphorothioate backbone. CpG ODN has a broad adjuvant effect which includes stimulation of B cells to proliferate, secrete immunoglobulin (Ig), cytokines (IL-6, IL-12), and to be protected from apoptosis (4–6); enhancement of expression of class II MHC and B7 costimulatory molecules (7,8); and direct activation of monocytes, macrophages, and dendritic cells to secrete various cytokines and chemokines that can provide T help (6–9). CpG ODN induce a Th1-like pattern of cytokine production dominated by IL-12 and IFN- γ with little secretion of Th2 cytokines (6). In general, Th1-like responses are associated with enhanced levels of neutralizing and opsonizing antibodies, CTL activity, and Th1 cytokines (IL-12, IFN- γ , TNF- β), and are desirable for protection against, or treatment of, certain diseases caused by intracellular bacterial and viral pathogens, including HBV. Furthermore, since the increased incidence of asthma and allergy in developed countries has been associated with decreased exposure to bacterial infections that stimulate development of Th1-biased immunity, it is possible that the use of CpG ODN as adjuvant in vaccines may also help reduce the growing incidence of asthma.

2. CpG DNA IS AN EFFECTIVE ADJUVANT WITH HEPATITIS B VACCINES

We have developed the use of CpG ODN as an adjuvant in HBV vaccines and have shown that when delivered parenterally (IM) to mice (7,10), nonhuman primates (11,12) and humans (13), a vaccine containing HBsAg plus alum and CpG ODN is superior to a vaccine that contains HBsAg with only alum. Furthermore, where alum is strongly Th2-biased and thus induces poor cell mediated immunity, CpG ODN preferentially induces Th1 cytokines, and results in both strong humoral and cell-mediated responses including cytotoxic T lymphocytes (CTL) in mice.

In 1998, an outbreak of HBV on the island of Borneo (Kalimantan) among orangutans being rehabilitated under the Wanariset Orangutan Rehabilita-

tion Program for reintroduction to the wild, caused several deaths and prompted the Indonesian government to initiate a vaccination program. Upon vaccination with a commercial vaccine (Engerix-B), it was found that orangutans are hypo-responders, with less than 15% seroconversion after two doses. However, addition of CpG ODN (50 µg) increased seroconversion rates after two doses to 100%, with no apparent adverse effects (11). This ongoing project has resulted in a more rapid immunization of orangutans at risk and allowed their faster reintroduction to the rain forest. Furthermore, our findings with orangutans provided valuable information regarding the potential use of CpG ODN in humans since orangutans are genetically very similar to humans. These findings also suggested that the addition of CpG ODN to an HBV vaccine containing alum may improve its efficacy in hypo-responsive individuals. We have now initiated a human clinical trial using CpG ODN as an adjuvant for the Engerix B vaccine for the prophylactic immunization of normal healthy volunteers. Interim results show HBsAg-specific antibody responses were significantly higher and appeared earlier in CpG ODN treated than control subjects, and that CpG ODN was generally well tolerated, both locally and systemically (13).

CpG ODN has also been shown to be highly effective in very young mice for inducing or enhancing humoral and cell-mediated immune responses against HBsAg (14). Furthermore, not only does CpG ODN elicit higher responses at an earlier age than does HBsAg alone or with alum, but these responses are more Th1-like. This is of particular interest since the neonatal immune response is strongly Th2 biased with poor cell-mediated immunity and therefore cannot effectively prevent or control many infectious diseases. In adult mice, we have found that the addition of CpG ODN to an HBV vaccine containing alum allows equivalent responses to be attained but with a 100-fold lower antigen dose than an HBV vaccine containing only alum as adjuvant (Comanita, Weeratna and Davis, unpublished observations). Therefore, it is possible that the addition of CpG ODN to current human vaccines may permit lower doses of antigen to be used, thereby reducing vaccine costs and decreasing the risk of antigen crossreactivity in multivalent vaccines. We have also demonstrated CpG to be an effective mucosal adjuvant in that oral or IN delivery to mice of HBsAg alone has no effect, but when combined with CpG ODN it elicits strong humoral and cell-mediated systemic immunity as well as mucosal immune responses at both local and distant sites (15–18). Although mucosal immune responses do not appear necessary for protection against HBV infection, the possibility to deliver HBV vaccines by an easy and noninvasive means is attractive for mass prophylactic vaccination.

3. COMPARISON OF CpG ODN WITH OTHER PARENTERAL ADJUVANTS

A large variety of immunological adjuvants have been used to augment protective immune responses to coadministered antigens. Different adjuvants may influence:

1. The biological or immunological half-life of vaccine antigens,
2. Antigen uptake and presentation.
3. The production of immunomodulatory cytokines that may influence the development of Th1- or Th2-type immune responses (19).

Despite the large number of adjuvants used in animal models, most adjuvanted human vaccines still contain alum. Alum is effective for parenteral administration of proteins and peptides, however it generally generates a Th2-biased immune response to coadministered antigens with diminished Th1 responses (CTL, T-cell proliferation).

We have compared CpG ODN with a number of adjuvants that are currently used in animal research (Freund's complete adjuvant, *FCA*, Freund's incomplete adjuvant, *FIA*, Titermax® Gold), licensed for human use (alum), or are in clinical testing for humans (MPL) for their ability to augment humoral responses to HBsAg and for the degree of damage they caused in the injected muscle (10). The greatest increase in anti-HBs titers (>100× higher than with HBsAg alone) was with *FCA* or Titermax as adjuvant, but both of these caused severe damage in the injected muscle (Fig. 1). Strong adjuvant activity was also seen with *FIA*, MPL, and CpG ODN, which increased anti-HBs titers by 20–50 fold over HBsAg alone and caused only moderate (*FIA*) or mild (CpG ODN and monophosphoryl lipid A [*MPL*]) damage. The weakest adjuvant for augmenting antibody responses was alum, which also caused the least muscle damage, which was no greater than that caused by an injection of saline. When CpG ODN was combined with alum, MPL or *FIA*, a synergistic relationship was found, although CpG + MPL also increased muscle damage. Overall, highest anti-HBs titers with least muscle tissue damage were obtained with CpG + alum. This synergy was most likely owing to the depot effect of alum, which could greatly enhance the period of time that both the antigen and the CpG ODN, a highly soluble molecule, were available.

When antibody isotypes were used as an indication of the Th-bias of the responses induced by the different formulations in mice, (Th1, IgG2a >> IgG1; Th2, IgG1>>IgG2a), we found that HBsAg without adjuvant induces mixed Th1/Th2 responses, but addition of alum, *FIA* or Titermax Gold all change the responses to a strong Th2 bias, with virtually no IgG2a (10). In

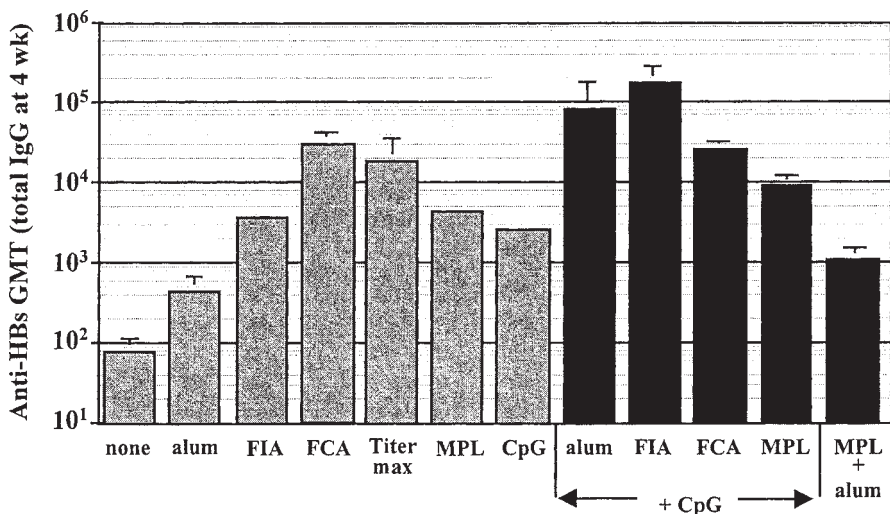


Fig. 1. Female BALB/c mice (6–8 wk, Charles River, Montreal, QC) were immunized under light anesthesia by im injection (50 μ L) into the left tibialis anterior (TA) muscle with 1 μ g HBsAg in PBS either alone (none) or with aluminum hydroxide (alum, Alhydrogel “85” Superfos Biosector, Vedbaek, Denmark, 25 μ g Al³⁺/ μ g HBsAg), Freund's Complete Adjuvant (FCA, Sigma, St. Louis, MO, 1:1 v/v), Freund's Incomplete Adjuvant (FIA, Sigma, 1:1 v/v), Titermax Gold (CytRx, Norcross, GA, 1:1 v/v), MPL (Ribi Immunochem Research Inc., Hamilton, MT, 50 μ g/animal), and/or CpG-containing oligodeoxynucleotides (CpG, ODN sequence #1826 TCCATGACGTTTCCTGACGTT, Coley Pharmaceutical Group, Wellesley, MA, 10 μ g/animal) as adjuvant. All mice were boosted in an identical manner at 4 wk. Each bar represents the group geometric mean titer (GMT) ($\pm$ SEM) of HBsAg-specific antibodies (anti-HBs), detected and quantified as previously described (7), in plasma taken 4 wk after second immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. (Reprinted from Weeratna RD, McCluskie MJ, Xu Y, and Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants, *Vaccine* 18, 1755–1762., [2000], with permission from Elsevier Science).

contrast, addition of FCA, MPL or CpG DNA, give mixed responses, with CpG DNA giving the most Th1-biased response of any adjuvant used alone. A similar strong Th1 bias of CpG ODN with parenteral delivery in the mouse was recently demonstrated by Kim et al., who compared 19 different immunological adjuvants with KLH conjugate vaccines containing two human cancer antigens (MUC1 peptide and GD3 ganglioside) and demonstrated CpG ODN to induce the most Th1 biased immune responses (20).

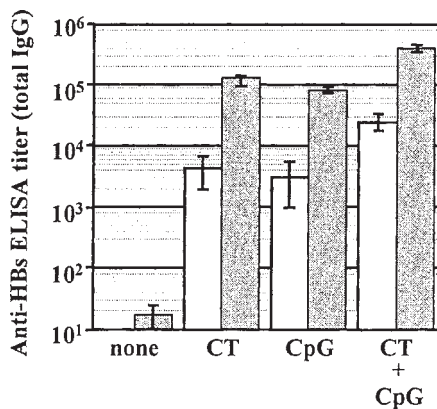


Fig. 2. Female BALB/c mice (6–8 wk) were immunized under light anesthesia by IN delivery of 1 µg (white bars) or 10 µg (gray bars) of HBsAg in PBS either alone (none), or with cholera toxin (CT) and/or CpG-containing oligodeoxynucleotides (CpG) as adjuvants, (1 µg each). Each bar represents the group mean ($\pm$ SEM) of HBsAg-specific antibodies (anti-HBs, total IgG), detected and quantified as previously described (7), in plasma taken 4 wk after immunization. Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. (Reprinted from McCluskie MJ and Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *The Journal of Immunology* 161, 4463–4466., (1998). Copyright 1998. The American Association of Immunologists.)

We have found that CpG ODN combined with almost any other adjuvant induces a very strong Th1-type response, even when that other adjuvant on its own has a strong Th2 bias, as do alum and FIA. CpG ODN preferentially induces Th1 cytokines such as IFN- γ and IL-12 (6), and its stronger Th1 immunostimulatory effects when combined with another adjuvant may depend on a depot effect of the coadministered adjuvant (alum, FIA). However, other mechanisms may be involved since a similar synergy and Th1 bias has also been demonstrated with parenteral codelivery of CpG and QS-21, a water-soluble saponin fraction, (21), and with mucosal delivery of CpG DNA together with cholera toxin, a potent Th2-type aqueous mucosal adjuvant (15).

4. COMPARISON OF CpG ODN WITH OTHER MUCOSAL ADJUVANTS

The most commonly used mucosal adjuvants in animal models are cholera toxin (CT), *Escherichia coli* heat labile enterotoxin (LT), and their

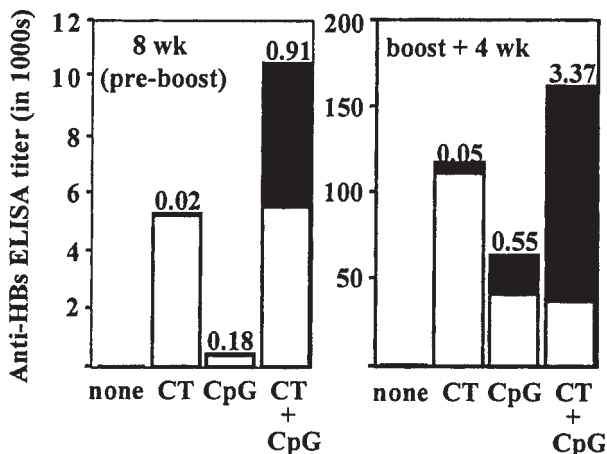


Fig. 3. Female BALB/c mice (6–8 wk) were immunized at 0 and 8 wk under light anesthesia by in delivery of 1 μ g of HBsAg in PBS either alone (none), or with cholera toxin (CT) and/or CpG-containing oligodeoxynucleotides (CpG) as adjuvants, (1 μ g each). Each bar represents the group mean ($\pm$ SEM) of HBsAg-specific antibodies (anti-HBs) of IgG1 (white bars) or IgG2a (black bars) isotypes, detected and quantified as previously described (7), in plasma taken eight wk post prime (left panel) or 4 wk post boost (right panel). Titers were defined as the highest plasma dilution resulting in an absorbance value two times that of nonimmune plasma, with a cut-off value of 0.05. The numbers above each bar indicate the IgG2a to IgG1 ratio, with a value >1 indicating a more Th1-like response. (Reprinted from McCluskie MJ and Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *The Journal of Immunology* 161, 4463–4466., (1998). Copyright 1998. The American Association of Immunologists.)

derivatives (22). With both intranasal and oral delivery of HBsAg we have shown CpG ODN to have equivalent adjuvant activity to native CT and LT, but with a lower toxicity (15–18) (Fig. 2). CpG ODN gave better or equal overall antigen-specific immune responses with both IN and oral routes than the nontoxic B subunits of CT or LT (CTB and LTb, respectively) (17), or five different genetically detoxified LT derivatives (McCluskie et al., unpublished observations). Similar findings have also been demonstrated with other antigens such as β -galactosidase (23,24), tetanus toxoid (18,25) or influenza (25) antigens. The immunostimulatory effects of CpG ODN at mucosal surfaces can be seen after delivery of single or multiple antigens and antigen-specific IgA can be detected in both local and distant mucosal secretions (25). Thus mucosal immune responses are not restricted to the site of application but were also present at distant mucosal surfaces.

However, whereas CT, LT, and their derivatives preferentially induce Th2-type responses (IgG1>IgG2a), with mucosal delivery CpG ODN has been shown to be Th1-like, even when given with strongly Th2 biased antigens (15–18,25,26). In addition, CpG DNA can overcome the Th2 bias of other mucosal adjuvants. For example, intranasal coadministration of CpG ODN with the strongly Th2-biased CT induced a mixed Th1/Th2 response that became even more Th1 after boost (15) (Fig. 3). A similar Th1 bias has been seen when CpG ODN was coadministered with LT (17), LTR72 (27), LTK63 (17), and five other different genetically detoxified LT derivatives (McCluskie et al., unpublished observations). Despite the Th1 bias of CpG ODN with mucosal delivery, high secretory IgA titers are also induced (15,18,25). IgA has historically been associated with Th2 responses, however, these results confirm reports that mucosal IgA can be synthesized in the context of either Th1 or Th2-biased systemic immune responses (28,29).

5. CONCLUSIONS

The addition of CpG ODN to existing HBV vaccines may lead to the development of an HBV vaccine that is effective with fewer doses and thus less expensive. This would facilitate mass immunization programs and increase the accessibility of HBV vaccination in poor areas of the world. Furthermore, the addition of CpG ODN may result in a more rapid protective immune response that is highly desirable in at risk populations. Furthermore, the potential to induce Th1-like immune responses that include CTLs may allow CpG containing vaccines to be used therapeutically in HBV chronic carriers. Thus, a great potential exists for CpG ODN in HBV treatment or prophylaxis.

REFERENCES

1. World Health Organization. (1996) Expanded Programme on Immunization. *WHO Update* 31.
2. Stephenne, J. (1988) Recombinant versus plasma-derived hepatitis B vaccines: issues of safety, immunogenicity and cost-effectiveness. *Vaccine* **6**, 299–303.
3. World Health Organization. (1998) Hepatitis B Fact sheet WHO/204.
4. Krieg, A. M., Yi, A. K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
5. Yi, A. K., Klinman, D. M., Martin, T. L., Matson, S., and Krieg, A. M. (1996). Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol.* **157**, 5394–5402.
6. Klinman, D., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN- γ . *Proc. Natl. Acad. Sci. USA* **93**, 2879.

7. Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J., and Krieg, A. M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant Hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
8. Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W., and Wagner, H. (1998). Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
9. Halpern, M. D., Kurlander, R. J., and Pisetsky D. S. (1996). Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell Immunol.* **167**, 72.
10. Weeranta, R. D., McCluskie, M. J., Xu, Y., and Davis, H. L. (2000) CpG DNA is a novel non-toxic adjuvant which induces stronger immune responses than many conventional adjuvants *Vaccine* **18**, 1755–1762.
11. Davis, H. L., Suparto, I., Weeratna, R., et al. (2000) CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. *Vaccine* **18**, 1920–1924.
12. Hartmann, G., Weeratna, R. D., Ballas, Z. K., et al. (2000). Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J. Immunol.* **164**: 1617–24.
13. Davis, H. L., Cooper, C. L., Morris, M. L., Elfer, S. M., Cameron, D. W., and Heathcote, J. (2000). CpG ODN is safe and highly effective in humans as adjuvant to HBV vaccine: Preliminary results of Phase I trial with CpG ODN 7909. *The Third Annual Conference on Vaccine Research* **S25**, 47.
14. Brazolot Millan, C. L., Weeratna, R., Krieg, A. M., Siegrist, C. A., and Davis, H. L. (1998) CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl. Acad. Sci. USA* **95**, 15,553–15,558.
15. McCluskie, M. J. and Davis, H. L. (1998) CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* **161**, 4463–4466.
16. McCluskie, M. J. and Davis, H. L. (1999) CpG DNA as mucosal adjuvant. *Vaccine* **18**, 231–237.
17. McCluskie, M. J., Weeratna, R. D. and Davis, H. L. (2000) Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. *Mol. Med.* **6**, 867–877.
18. McCluskie, M. J., Weeratna, R. D., Krieg, A. M., Davis, H. L. (2000) CpG DNA is an effective oral adjuvant to protein antigens in mice. *Vaccine* **19**, 950–957.
19. Vogel, F. R. (1998) Adjuvants in perspective. *Dev. Biol. Stand.* **92**, 241–248.
20. Kim, S. K., Ragupathi, G., Musselli, C., Choi, S. J., Park, Y. S., and Livingston, P. O. (1999) Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. *Vaccine* **18**, 597–603.
21. Kim, S. K., Ragupathi, G., Cappello, S., Kagan, E., Livingston, P.O. (2000) Effect of immunological adjuvant combinations on the antibody and T-cell response to vaccination with MUC1-KLH and GD3-KLH conjugates. *Vaccine* **19**, 530–537.

22. Freytag, L. C., Clements, J. D. Bacterial toxins as mucosal adjuvants. (1999) *Curr. Top Microbiol. Immunol.* **236**, 215–236.
23. Horner, A. A., Ronaghy, A., Cheng, P. M., et al. (1998) Immunostimulatory DNA is a potent mucosal adjuvant. *Cell Immunol.* **190**, 77–82.
24. Horner, A. A. and Raz, E. (2000) Immunostimulatory sequence oligodeoxynucleotide: A novel mucosal adjuvant. *Clin. Immunol.* **95**, S19–29.
25. McCluskie, M. J. and Davis, H. L. (2000) Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine* **19**, 413–422.
26. McCluskie, M. J., Wen, Y. M., Di, Q. and Davis, H. L. (1998) Immunization against hepatitis B virus by mucosal administration of antigen-antibody complexes. *Viral Immunol.* **11**(4), 245–252
27. Olszewska, W., Partidos, C. D., and Steward, M. W. (2000) Antipeptide antibody responses following intranasal immunization: effectiveness of mucosal adjuvants. *Infect Immun.* **68**, 4923–4929.
28. Marinaro, M., Boyaka, P. N., Finkelman, F. D., et al. (1997) Oral but not parenteral interleukin (IL)-12 redirects T helper 2 (Th2)-type responses to an oral vaccine without altering mucosal IgA responses. *J. Exp. Med.* **185**, 415–427.
29. Marinaro, M., Boyaka, P. N., Jackson, R. J., et al. (1999) Use of intranasal IL-12 to target predominantly Th1 responses to nasal and Th2 responses to oral vaccines given with cholera toxin. *J. Immunol.* **162**, 114–121.

Immunostimulatory DNA-Based Immunization

Hope for an HIV Vaccine?

**Sandip K. Datta, Anthony A. Horner,
Kenji Takabayashi, and Eyal Raz**

1. INTRODUCTION

Despite extensive efforts, an effective vaccine against HIV remains elusive. Major advances in treatments for HIV infection and AIDS have been made, but these treatments have limitations and are not widely available to the vast majority of infected people in developing countries. A safe and effective vaccine, with potential for both preventive and therapeutic use, remains the best hope for controlling the worldwide impact of HIV. The ultimate success of any particular HIV vaccine candidate is difficult to predict, owing to limitations of current animal models and lack of reliable laboratory correlates of protective immunity. However, to overcome HIV's enormous capabilities of immune evasion, a successful HIV vaccine will likely need to elicit multiple aspects of the humoral and cellular immune system (1,2).

A protective immune response against infectious pathogens relies on activation of innate and adaptive immunity. The innate immune system has evolved to recognize conserved structural motifs on microbial pathogens, termed pathogen-associated molecular patterns (PAMPs) (3). Recognition of PAMPs by antigen presenting cells (APCs), leads to enhanced phagocytosis, secretion of proinflammatory cytokines essential for reduction of the infectious load, and orchestration of an adaptive immune response, which includes humoral and cellular immunity against the infectious agent (3–5).

Recent studies have emphasized the central role of dendritic cells in initiating adaptive immune responses after exposure to PAMPs in their microenvironment (6). Because the response of cells of innate immunity, such as dendritic cells, to PAMPs is deeply rooted in evolution, it is likely that PAMPs act as natural adjuvants for the induction of protective immune

responses against the pathogens that contain them. This suggests an intriguing strategy for vaccine design: Combining a suitable PAMP with pathogen-derived antigens, to create immunogens that elicit protective immune responses in the immunized host.

Bacterial DNA fulfills the definition of a PAMP as it is recognized by the vertebrate innate immune system on the basis of immunostimulatory sequences (ISS), which are structurally defined by their content of unmethylated CpG motifs (5'-purine-purine/T-CpG-pyrimidine-pyrimidine-3') and are underrepresented in the vertebrate genome (7,8). ISS are potent activators of innate immunity, stimulating APCs to secrete cytokines that shape Th1-biased immunity (7,9,10), upregulate class I and class II major histocompatibility (MHC) and costimulatory molecules (11–13), and induce cross-presentation of soluble antigen on MHC class I for presentation to CD8+ T cells (14). This ability of ISS to elicit multiple aspects of the immune response, coupled with the capability to produce large quantities of synthetic oligodeoxynucleotides (ODNs) containing ISS and the relatively low toxicity of these synthetic ODNs when compared to other PAMPs such as LPS, make ISS an attractive reagent for use in vaccines.

The following discussion will review currently available data on the use of ISS-based immunization schemes for the development of HIV vaccines. In particular, it will evaluate the capability of ISS-based vaccines to elicit the following desirable attributes of an ideal HIV vaccine:

1. Neutralizing antibody responses,
2. Th1-biased T helper cell responses,
3. Secretion of β -chemokines,
4. Cytotoxic T lymphocyte (CTL) responses,
5. Immunity at mucosal sites,
6. Efficacy in the absence of CD4+ T cells.

2. RESULTS

The following data evaluate the ability of ISS to induce immune parameters desirable for an HIV vaccine. Immune parameters were measured after immunizing Balb/c mice on three occasions spaced two weeks apart with a model HIV envelope antigen, gp120, co-administered (10 μ g gp120 + 50 μ g ISS) or directly conjugated (10 μ g based on gp120 content; ISS content was approx 1.3 μ g based on protein:ODN ratio of 3:1) with phosphorothioate oligonucleotides containing ISS (5'-TGACTGTGAACGTTTCGAGATGA-3'). For comparison, control mice were immunized with gp120 alone, gp120 + mODN (mutated ODN: 5'-TGACTGTGAACCTTAGAGATGA-3'), or gp120:mODN conjugate. Synthesis of the gp120:ODN conjugates has been

previously described (15). As is further discussed in Section 2.1., gp120 is a weak immunogen, but these experiments provide proof-of-principle for the application of ISS-based immunization strategies to generate improved immunity to better HIV-derived antigens as they are identified.

2.1. Th1 Immunity and Chemokine Secretion

Most anti-viral vaccines in use today elicit an effective neutralizing antibody response that helps prevent infection of host cells. However, establishing an effective neutralizing antibody response in HIV vaccine models has been difficult, at least in part due to epitope inaccessibility of the gp120 HIV envelope protein (16,17). In addition, the neutralizing antibody responses against T cell-adapted R4 virus used in laboratory studies have been ineffective against macrophage-tropic R5 virus found in primary isolates, making the generalizability of neutralizing responses unclear (18,19). Furthermore, antibody responses have the potential to enhance viral infectivity owing to antibody-mediated viral uptake by immune cells (20–22). Neutralizing antibody responses to ISS-based immunization with gp120 have been reported (15), although, based on the above principles, the clinical implications are unclear. However, the ability of ISS to augment antibody responses may predict its ability to augment neutralizing responses when used with an appropriate antigen that is capable of eliciting effective neutralizing antibodies. To assess the ability of the previously described vaccination reagents to augment humoral immune responses, antigen-specific total IgG and IgG2a (which is Th1-dependent) levels from serum collected 12 wk after initiation of intradermal (i.d.) immunization were measured (Table 1a) (23). Compared with controls, mice i.d. immunized with gp120 + ISS or gp120:ISS conjugate showed significantly higher levels of total IgG and IgG2a ($p < 0.001$). In addition, both gp120 + ISS coadministration and gp120:ISS immunization improved IgG1 responses relative to control immunizations (data not shown). Future studies with HIV-related antigens effective at eliciting neutralizing antibodies, will need to verify the ability of ISS to augment neutralizing antibody responses.

IFN γ production is a hallmark of Th1-biased immunity and contributes to protection against many viral infections (24,25). Therefore, the CD4+ T cell IFN γ response of immunized mice was determined by culture of splenocytes with gp120 and analysis of supernatants by ELISA (Table 1a) (23). IFN γ production was significantly higher for mice immunized with gp120 + ISS ($p < 0.05$) or gp120:ISS conjugate ($p < 0.001$) when compared with control mice. Furthermore, gp120:ISS conjugate was more effective at inducing an IFN γ response than gp120 + ISS ($p < 0.001$).

Table 1
ISS-Based gp120 Vaccines Elicit Antigen-Specific Immunoglobulin and Cytokine Responses

	a. Intradermal Immunization					b. Intranasal Immunization				
	gp120	gp120+mODN	gp120:mODN conjugate	gp120+ISS	gp120:ISS conjugate	gp120	gp120+mODN	gp120:mODN conjugate	gp120+ISS	gp120:ISS conjugate
IgG (Ux10 ³ /mL)	34 ± 93	47 ± 5	3.6 ± 2.3	202 ± 44 *	221 ± 20 *	0.3 ± 0.2	4.0 ± 2.6	n.d.	402 ± 47 **	488 ± 4.1 ** #
IgG2a (Ux10 ³ /mL)	0.9 ± 0.9	0.5 ± 0.1	n.d.	255 ± 74 *	280 ± 15**	n.d.	n.d.	0.1 ± 0.1	45 ± 22	289 ± 78 ** ##
Vaginal IgA (Ux10 ³ /mL)					1.2 ± 0.2	1.8 ± 0.5	0.1 ± 0.1	.3 ± 0.1	20 ± 5.8 *	19 ± 1.6 **
Fecal IgA (Ux10 ³ /mL)					12 ± 3.3	1.2 ± 0.3	1.9 ± 0.3	2.0 ± 0.6	37 ± 8.5 *	37 ± 9.7 *
IFNγ (pg/mL)	55 ± 32	142 ± 15	11 ± 11	2934 ± 528	13216 ± 996 **##	1.0 ± 1.0	25 ± 25	n.d.	2291 ± 432 **	3004 ± 357 **
MIP1α (pg/mL)	n.d.	n.d.	237 ± 43	203 ± 16	908 ± 142 **##	10 ± 5.2	177 ± 77	1.0 ± 0.7	280 ± 46	1343 ± 498 *#
MIP1β (pg/mL)	n.d.	n.d.	311 ± 141	3273 ± 755 *	4958 ± 51 **##	47 ± 28	906 ± 407	n.d.	2550 ± 957 *	2476 ± 587 *

Female BALB/c mice were immunized intradermally or intranasally on days 0, 14, and 28 with gp120 (10 µg) alone, gp120+mODN (50 µg), gp120:mODN conjugate (10 µg based on gp120 content), gp120+ISS (10 µg), or gp120:ISS conjugate (10 µg based on gp120 content). gp120-specific serum IgG, serum IgG2a, and vaginal and fecal IgA, represented in relative units based on comparison with respective high titer standards, were determined at wk 12 by ELISA. gp120-specific IFNγ, MIP1α, and MIP1β production from splenocytes restimulated in vitro with gp120 protein was determined by ELISA. Results reflect averages of four mice per group and are representative of three independent experiments. Results are reported as mean ± S.E.M. Groups whose means are significantly different from gp120-immunized mice are denoted with a * ($p < 0.05$) or ** ($p < 0.001$). Groups whose means are significantly higher than gp120+ISS-immunized mice are denoted with a # ($p < 0.05$) or ## ($p < 0.001$). n.d. denotes levels that were nondetectable by ELISA.

The CCR5 chemokine receptor acts as a coreceptor for HIV entry into cells (26–30) and competitive inhibition of this virus/coreceptor interaction by CCR5-specific β -chemokines (MIP1 α , MIP1 β , and RANTES) may inhibit the intercellular spread of HIV, and the natural progression of the infection to AIDS (31). The ability of ISS to induce β -chemokine production from macrophages in an antigen-independent manner (Horner, A. A., Datta, S. K. and Raz, E. unpublished observations) led to the investigation of whether ISS-based vaccines could elicit their antigen-specific production. Antigen-specific secretion of MIP1 α , MIP1 β , and RANTES was assessed by ELISA of supernatants from splenocytes cultured with gp120 (Table 1a) (23). Mice immunized with gp120:ISS conjugate demonstrated significantly stronger MIP1 α and MIP1 β responses than controls ($p < 0.001$) or gp120 + ISS-immunized mice ($p < 0.001$ for MIP1 α and $p < 0.05$ for MIP1 β). However, whereas less effective than gp120:ISS conjugate, gp120 + ISS coadministration also elicited significant MIP1 β , but not MIP1 α , production. Interestingly, while significant levels of RANTES have been observed upon stimulation of APCs by ISS *in vitro*, gp120-specific RANTES production was not appreciably induced above background levels in this series of experiments (data not shown).

2.2. Mucosal Immune Responses

Protection against HIV infection is likely to require immunity at mucosal sites as

1. Its spread is principally by sexual transmission,
2. The intestinal mucosa represents an important site for the initial replication of the virus.
3. Mucosal immunity is important for protection against mucosal challenge in published models of HIV viral infection (32,33).

As mucosal immunity is best elicited by vaccine delivery to mucosal sites (33,34), the immunization reagents described in the previous sections were administered intranasally (i.n.) to mice at the same doses used for i.d. immunization, and both systemic and mucosal immune parameters were measured (Table 1b) (23). Similar to i.d. immunization, i.n. immunization with gp120 + ISS or gp120:ISS conjugate elicited significantly higher levels of serum IgG and IgG2a than controls ($p < 0.001$). Furthermore, i.n. immunization with gp120 + ISS or gp120:ISS conjugate also induced a vigorous secretory IgA response detected in vaginal washes and fecal samples ($p < 0.001$ vs controls). In contrast, i.d. immunization with these reagents failed to elicit a significant mucosal IgA response. Finally, i.n. immunization with either gp120 + ISS or gp120:ISS conjugate elicited significantly more gp120-spe-

cific IFN γ , MIP1 α , and MIP1 β production than control immunizations ($p < 0.001$ for IFN γ , $p < 0.05$ for MIP1 α and MIP1 β).

2.3. Systemic and Mucosal CTL Activity

Since an effective CD8+ CTL response is important in preventing and controlling HIV infection (2,35–38), the ability of ISS-based gp120 immunization to elicit antigen-specific CTL activity was determined (23). Both i.d. (Fig. 1a) and i.n. (Fig. 1b) administration of gp120:ISS conjugate elicited similar levels of high specific lysis in splenic CTL assays. However, whereas i.d. administration of gp120 + ISS elicited CTL activity that was similar to the conjugate, i.n. administration of gp120 + ISS elicited a significantly lower CTL response ($p < 0.05$). In addition to systemic CTL activity, in gp120:ISS conjugate delivery, and to a much lesser extent gp120 + ISS coadministration, induced mucosal CTL responses as measured with lamina propria (Fig. 1c) and Peyer's patch lymphocytes (Fig. 1d). However, consistent with the poor secretory IgA response seen after systemic vaccination, both i.d. gp120 + ISS and gp120:ISS conjugate immunizations induced only weak CTL responses at these mucosal sites.

2.4. MHC Class I-Restricted Cytokine and Chemokine Responses

The cytokine and chemokine data described in the previous Subheadings reflect MHC class II-dependent responses, as intact gp120 protein was used to stimulate cells. Cytokine and chemokine secretion by CD8+ T cells, in addition to CTL responses, are important for controlling HIV infection, whereas CD4+ T-cell deficiency is a characteristic feature of AIDS (31,39). Therefore, the ability of ISS-based vaccines to induce cytokine and chemokine responses from CD8+ T cells was investigated [23]. Splenocytes from immunized mice were restimulated in vitro with MHC class I (H2^d)-restricted gp120 peptide, and cytokine and chemokine production in culture supernatants was subsequently determined by ELISA. Mice immunized i.d. with either gp120 + ISS or gp120:ISS conjugate demonstrated significant CD8+ T-cell production of IFN γ , MIP1 α , and MIP1 β compared to control immunized mice (Table 2). Similar results were seen with in gp120 + ISS and gp120:ISS conjugate vaccination (data not shown).

2.5. CD4+ T-Cell-Independent Class I-Restricted Cytokine, Chemokine, and CTL Responses

During the course of HIV infection, CD4+ T cells are depleted. Therefore, it would be important for a therapeutic AIDS vaccine to elicit robust immunity in the absence of CD4+ T cells. The ability of ISS-based vaccines

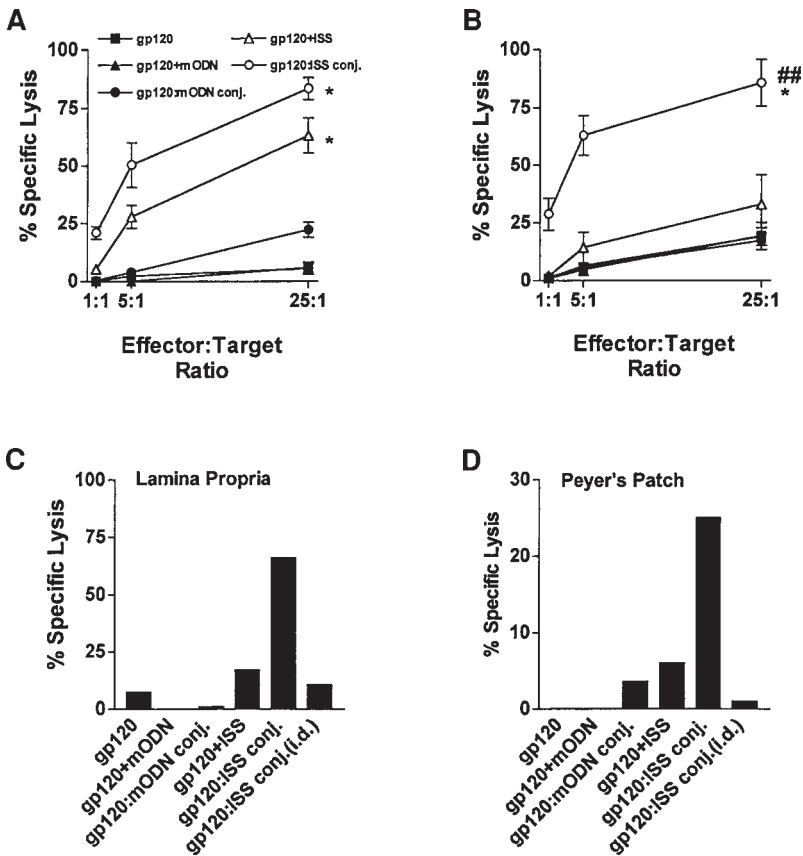


Fig. 1. ISS-based gp120 vaccines elicit splenic and mucosal CTL activity. gp120-specific splenic CTL activity was determined using class I-restricted gp120 peptide-loaded P815 target cells 12 wk after the initiation of (A), intradermal or (B), intranasal immunization. Splenic CTL data reflect averages of four mice per group and are representative of three independent experiments. Error bars indicate S.E.M. Statistical significance is denoted as in Table 1. Mucosal CTL activity from (C) lamina propria and (D) Peyer's patch lymphocytes was determined 12 wk after initiation of intranasal or intradermal (i.d.) immunization. Mucosal CTL responses at an effector:target ratio of 25:1 are shown. Mucosal CTL results were obtained by pooling lymphocytes from four mice per group and are representative of three independent experiments.

to elicit cytokine, chemokine, and CTL responses from CD8+ T cells, led to the investigation of whether these responses required CD4+ T cell help. Previous investigations have demonstrated that ovalbumin:ISS (OVA:ISS) conjugate vaccination induces equivalent CTL responses in CD4-knockout and

Table 2
ISS-based gp120 Vaccines Elicit MHC Class I-Restricted Cytokine Responses that are Independent of CD4+ T cells

	gp120	gp120+mODN	gp120:mODN conjugate	gp120+ISS	gp120:ISS conjugate	gp120:ISS conjugate (CD4-depleted)
IFN γ (pg/mL)	n.d.	n.d.	n.d.	1010 $\pm$ 134 *	1472 $\pm$ 344 **	1584 $\pm$ 534 **
MIP1 α (pg/mL)	n.d.	n.d.	n.d.	119 $\pm$ 53 *	122 $\pm$ 19 *	259 $\pm$ 18 *
MIP1 β (pg/mL)	n.d.	n.d.	12 $\pm$ 11	543 $\pm$ 142 *	1228 $\pm$ 200 ***##	1446 $\pm$ 17 **#

Splenocytes from nondepleted or CD4+ T cell-depleted mice immunized intradermally as in Table 1 were restimulated in vitro with MHC class I-restricted gp120 peptide. Cytokine levels in the supernatant were measured by ELISA. Results reflect averages of four mice per group and are representative of three independent experiments. Results are reported as mean $\pm$ S.E.M. Statistical significance and abbreviations are denoted as in Table 1.

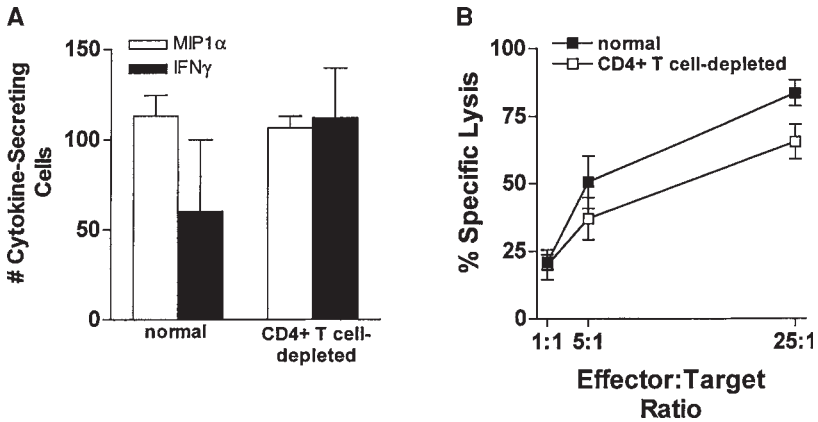


Fig. 2. CTL activity elicited by gp120:ISS vaccination is independent of CD4+ T cells. Splenocytes were obtained from untreated and anti-CD4 mAb-treated mice 12 wk after the initiation of intradermal immunization with gp120:ISS conjugate as described in Table 1. Effective CD4+ T cell depletion was confirmed by flow cytometry (<1% peripheral blood and splenic CD4+ T cells compared with nondepleted mice) and lack of MHC class II-restricted cytokine secretion upon restimulation of splenocytes with gp120 protein (data not shown). **(A)** The number of splenocytes per 1×10^5 CD8+ T cells secreting MIP1 α and IFN γ in a MHC class I-restricted fashion from wild-type and CD4-depleted mice, was determined by ELISPOT. **(B)** Splenic CTL activity in immunized wild-type and CD4+ T cell-depleted mice was determined. Data reflect averages of four mice per group. Error bars indicate S.E.M. Statistical significance is denoted as in Table 1. Similar results were obtained with intranasal immunization (data not shown).

wild-type mice, whereas the CTL response in CD4-knockout mice immunized with OVA + ISS is compromised (14). Therefore, gp120:ISS conjugate was used to i.d. immunize wild type and CD4+ T cell-depleted mice in order to compare their CD8+ T-cell responses (23). Splenocytes from gp120:ISS conjugate-immunized, CD4+ T-cell depleted mice that were restimulated with a class I-restricted gp120 peptide demonstrated a retained ability to secrete antigen-specific IFN γ , MIP1 α , and MIP1 β relative to splenocytes from immunized mice that were not CD4+ T-cell depleted (Table 2). Furthermore, by ELISPOT analysis, CD4+ T-cell depleted and non-depleted mice immunized with gp120:ISS conjugate had equivalent frequencies of CD8+ T cells producing IFN γ and MIP1 α in response to incubation with a class I-restricted gp120 peptide (Fig. 2A). Consistent with these results, antigen-specific CTL activity was also retained in CD4+ T-cell depleted mice (Fig. 2B). As expected, restimulation of splenocytes from immunized CD4+ T-cell depleted mice with gp120 protein failed to elicit

cytokine or chemokine responses (data not shown). Furthermore, CD4+ T- cell depleted mice were unable to generate a detectable antibody response after gp120:ISS conjugate immunization (data not shown). Similar to i.d. immunized mice, CD4+ T-cell depleted mice immunized i.n. with gp120:ISS conjugate also showed retained CD8+ T-cell responses, including CTL responses at both systemic and mucosal sites (data not shown).

3. DISCUSSION

The wide variety of immune responses elicited with ISS-based immunization highlights the potential for harnessing the immunostimulatory properties of PAMPs in order to develop more effective HIV vaccines. ISS-based gp120 vaccines induce robust antigen-specific antibody, Th1 cytokine, and CTL responses. In addition, this chapter highlights the ability of ISS-based vaccines to stimulate MHC class I- and II-restricted production of CCR5-specific β -chemokines such as MIP1 α and MIP1 β , which have been shown to protect against cellular invasion by HIV (26–31). Furthermore, the presented studies establish that ISS-based immunization elicits gp120-specific cytokine, chemokine, and CTL responses from CD8+ T cells in CD4+ T cell-deficient mice, suggesting that this vaccination strategy could have clinical utility even in CD4+ T-cell depleted AIDS patients for whom CD8+ T cells are likely to play a crucial role in controlling infection (2,31,35–39).

The ability of i.n. ISS-based immunization to induce mucosal immunity, consistent with previously published data using other antigens (40–42), represents another attractive feature of this vaccination strategy, as HIV is transmitted primarily by mucosal routes, and the presence of mucosal IgA in human vaginal secretions has been correlated with resistance to infection in commercial sex workers (43). Intranasal immunization, especially with the gp120:ISS conjugate, also effectively elicits systemic immune responses, although certain immune parameters are not as robust as with i.d. vaccination. It remains to be seen whether delivery of an ISS-based HIV vaccine by mucosal routes alone is sufficient, or whether a combination of systemic and mucosal immunizations will be needed to optimize systemic and mucosal immunity.

Similar to previously described results with other antigens (14,15,44), the presented data demonstrates that i.d. gp120:ISS conjugate vaccination induces a stronger immune response than coadministration of an equivalent dose of gp120 with 40-fold higher amounts of unconjugated ISS, and further establishes the improved immunogenicity of gp120:ISS conjugate for mucosal vaccination. The immunologic potency of the gp120:ISS conjugate relative to gp120 mixed with ISS can best be explained by reviewing the known mechanisms of action of ISS. As previously discussed, ISS, like other

PAMPs, exert their effects mainly on APCs, such as macrophages and dendritic cells, stimulating them to secrete cytokines and upregulate antigen processing and costimulatory molecules. ISS-activated APCs are thus primed to instruct lymphocytes and efficiently promote adaptive immune responses (5,10,45,46). The gp120:ISS conjugate physically colocalizes the antigen to these ISS-activated APCs. Although without ISS conjugation, only a fraction of ISS-activated APCs will encounter, process, and present the antigen for the orchestration of the subsequent adaptive immune response. The presented results suggest that for difficult-to-elicited immune responses, such as the induction of systemic immunity with mucosal immunization, or the induction of immunity in the absence of CD4⁺ T cells, gp120:ISS conjugate vaccination offers a distinct advantage over coadministration of gp120 and ISS.

Previous investigations have shown that OVA:ISS conjugate elicits equivalent systemic CTL responses in CD4-knockout and wild-type mice (14). The investigations presented here expand on this observation, demonstrating that gp120:ISS conjugate vaccination elicits equivalent systemic and mucosal CD8⁺ T cell effector responses in mice that have been depleted of CD4⁺ T cells compared with non-depleted mice. These CD8⁺ T-cell effector responses include cytokine and chemokine secretion, as well as CTL responses. The level of cytokines and chemokines secreted by CD8⁺ T cells is less than that seen with CD4⁺ T cell responses, but this CD8⁺ T cell response may be important, especially in states of CD4⁺ T-cell depletion such as AIDS. ELISPOT investigations further establish that CD4⁺ T-cell depletion does not reduce the frequency of CD8⁺ T cells that are generated in response to gp120:ISS vaccination. These results raise intriguing mechanistic questions about how ISS exert their profound effects on CD8⁺ T cell immunity. Insight into the ability of ISS to license APCs to cross-present soluble antigen on MHC class I and activate CD8⁺ T cells is further discussed elsewhere in this publication. Cytokines induced by ISS, some of which are known to be involved in the Th1-biased immune response seen with ISS-based immunization, probably also play a role in the initiation and maintenance of the CD8⁺ T cell response. For example, IL-15, which is induced by ISS in an IFN α/β -dependent manner (Takabayashi, K. and Raz, E. unpublished observations) has been recently implicated in the proliferation of activated CD8⁺ memory T cells (47,48). Therefore, in addition to licensing APCs and allowing cross-presentation of antigen, ISS may create an appropriate cytokine milieu for the induction of vigorous CD8⁺ T cell immunity.

The potency of ISS-based immunization demonstrated in this report validates the concept of using PAMPs (i.e., activators of innate immunity) in

conjunction with antigens (i.e., activators of adaptive immunity) to elicit robust immune responses. Reports of enhanced immunity to certain microbial antigens delivered with PAMPs derived from other pathogens (49,50) confirm the viability of this approach for future vaccine development. These studies further demonstrate the potential for harnessing the innate immunostimulatory properties of PAMPs from one pathogen to improve immune responses to antigens from unrelated, immunologically evasive pathogens.

In summary, the data presented here demonstrate that ISS-based vaccines, such as the gp120:ISS conjugate, elicit a multifaceted immune response with characteristics thought to be important for protection against HIV. These include

1. An antigen-specific antibody response.
2. A Th1 cytokine response.
3. The secretion of CCR5-specific β -chemokines.
4. A CTL response.
5. Mucosal immunity.
6. A CD8+ T cell response that is independent of CD4+ T cell help.

These results add to a growing body of evidence suggesting that PAMPs such as ISS, and their conjugation to relevant antigens, provide a means for recruiting and activating the innate immune system, for the purposes of enhancing and diversifying the adaptive immune response to antigens from infectious agents such as HIV.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI47078 and AI40682 (E.R.), AI01490 (A.A.H.), 5 T32 AI07036-22 (S.K.D), a grant from the Elizabeth Glaser Pediatric AIDS Foundation (A.A.H.), and by Dynavax Technologies Corporation (E.R.)

REFERENCES

1. Nabel, G. J. (2001) Challenges and opportunities for development of an AIDS vaccine. *Nature* **410**, 1002–1007.
2. McMichael, A. J. and Rowland-Jones, S. L. (2001) Cellular immune responses to HIV. *Nature* 2001 **410**, 980–987.
3. Janeway, C. A., Jr. and Medzhitov, R. (1998) Introduction: the role of innate immunity in the adaptive immune response. *Semin Immunol*, 1998, **10**, 349–350.
4. Aderem, A. and Underhill, D. M. (1999) Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol.* **17**, 593–623.

5. Fearon, D. T. and Locksley, R. M. (1996) The instructive role of innate immunity in the acquired immune response. *Science* **272**(5258), 50–53.
6. Gallucci, S., Lolkema, M., and Matzinger, P. (1999) Natural adjuvants: endogenous activators of dendritic cells. *Nat Med.* **5**, 1249–1255.
7. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
8. Bird, A. P. (1993) Functions for DNA methylation in vertebrates. *Cold Spring Harb. Symp. Quant Biol.* **58**, 281–285.
9. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
10. Roman, M., Martin-Orozco, E., Goodman, J. S., Nguyen, M. D., Sato, Y., Ronaghy, A., Kornbluth, R. S., Richman, D. D., Carson, D. A., and Raz, E. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
11. Bauer, M., Heeg, K., Wagner, H., and Lipford, G.B. (1999) DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology* **97**, 699–705.
12. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M. D., Kornbluth, R.S., and Raz, E., (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.
13. Sparwasser, T., Koch, E. S., Vabulas, R. M., Heeg, K., Lipford, G. B., Ellwart, J. W., and Wagner, H. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
14. Cho, H. J., Takabayashi, K., Cheng, P. M., Nguyen, M. D., Corr, M., Tuck, S., and Raz, E. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514.
15. Tighe, H., Takabayashi, K., Schwartz, D., Van Nest, G., Tuck, S., Eiden, J. J., Kagey-Sobotka, A., Creticos, P. S., Lichtenstein, L. M., Spiegelberg, H. L., and Raz, E. (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* **106**(1 Pt 1), 124–134.
16. Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., and Hendrickson, W. A. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648–659.
17. Wyatt, R., Kwong, P. D., Desjardins, E., Sweet, R. W., Robinson, J., Hendrickson, W. A., and Sodroski, J. G. (1998) The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* **393**, 705–711.
18. Mascola, J. R., Snyder, S. W., Weislow, O. S., et al. (1996) Immunization with envelope subunit vaccine products elicits neutralizing antibodies against

- laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. *J. Infect. Dis.* **173**, 340–348.
19. Burton, D. R. (1997) *A vaccine for HIV type 1: the antibody perspective. Proc. Natl. Acad. Sci. USA* **94**, 10,018–10,023.
 20. Mascola, J. R., Mathieson, B. J., Zack, P. M., Walker, M. C., Halstead, S. B., and Burke, D. S. (1993) Summary report: workshop on the potential risks of antibody-dependent enhancement in human HIV vaccine trials. *AIDS Res. Hum. Retroviruses* **9**, 1175–1184.
 21. Morens, D. M. (1994) Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clin. Infect. Dis.* **19**, 500–512.
 22. Burke, D. S. (1992) Human HIV vaccine trials: does antibody-dependent enhancement pose a genuine risk? *Perspect Biol. Med.* **35**, 511–530.
 23. Horner, A. A., Datta, S. K., Takabayashi, K., et al. (2001) Immunostimulatory dna-based vaccines elicit multifaceted immune responses against hiv at systemic and mucosal sites. *J. Immunol.* **167**, 1584–1591.
 24. Guidotti, L. G. and Chisari, F. V. (1996) To kill or to cure: options in host defense against viral infection. *Current Opinion in Immunology*, **8**, 478–483.
 25. van den Broek, M. F., Müller, U., Huang, S., Zinkernagel, R. M., and Aguet, M. (1995). Immune defence in mice lacking type I and/or type II interferon receptors. *Immunological Reviews*. **148**, 5–18.
 26. Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1996) CC CKR5: a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955–1958.
 27. Choe, H., Farzan, M., Sun, Y., et al. (1996) The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135–1148.
 28. Deng, H., Liu, R., Ellmeier, W., et al. (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661–666.
 29. Doranz, B. J., Rucker, J., Yi, Y., et al. (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the beta- chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149–1158.
 30. Dragic, T., Litwin, V., Allaway, G. P. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC- CKR-5. *Nature* **381**, 667–673.
 31. Cocchi, F., DeVico, A. L., Garzino-Demo, A., Arya, S. K., Gallo, R. C., and Lusso, P. (1995) Identification of RANTES, MIP-1 α , and MIP-1 β as the major HIV- suppressive factors produced by CD8+ T cells. *Science* **270**, 1811–1815.
 32. Veazey, R. S., DeMaria, M., Chalifoux, L. V. et al. (1998) Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* **280**, 427–431.
 33. Belyakov, I. M., Ahlers, J. D., Brandwein, B. Y. et al. (1998) The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. *J. Clin. Invest.* **102**, 2072–2081.

34. Gallichan, W. S. and Rosenthal, K. L. (1996) Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. *J. Exp. Med.* **184**, 1879–1890.
35. McMichael, A. (1998) T cell responses and viral escape. *Cell* **93**, 673–676.
36. Musey, L., Hughes, J., Schacker, T., Shea, T., Corey, L., and McElrath, M. J. (1998) Cytotoxic-T-cell responses, viral load, and disease progression in early human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **337**, 1267–1274.
37. Ogg, G. S., Jin, X., Bonhoeffer, S. (1998) Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* **279**, 2103–2106.
38. Yang, O. O., Kalams, S. A., Trocha, A., et al. (1997) Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J. Virol.* **71**, 3120–3128.
39. Walker, C. M., Moody, D. J., Stites, D. P., and Levy, J. A. (1986) CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. *Science* **234**, 1563–1536.
40. Horner, A. A., Ronaghy, A., Cheng, P. M. et al. (1998) Immunostimulatory DNA is a potent mucosal adjuvant. *Cell Immunol.* **190**, 77–82.
41. McCluskie, M. J. and Davis, H. L. (1998) CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* **161**, 4463–4466.
42. Moldoveanu, Z., Love-Homan, L., Huang, W. Q., and Krieg, A. M. (1998) CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* **16**, 1216–1224.
43. Kaul, R., Trabattoni, D., Bwayo, J. J., et al. (1999) HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. *Aids*, **13**, 23–29.
44. Shirota, H., Sano, K., Kikuchi, T., Tamura, G., and Shirato, K. (2000) Regulation of murine airway eosinophilia and Th2 cells by antigen- conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J. Immunol.* **164**, 5575–5582.
45. Davis, H. L., Weeratna, R., Waldschmidt, T. J., et al. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen [published erratum appears in J Immunol 1999 Mar 1;162(5):3103]. *J. Immunol.* **160**, 870–876.
46. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344.
47. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J., and Marrack, P., (2000) Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science* **288**, 675–678.
48. Zhang, X., Sun, S., Hwang, I., Tough, D. F., and Sprent, J. (1998) Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity* **8**, 591–599.

49. Toda, S., Ishii, N., Okada, E., et al. (1997) HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon-gamma antibody. *Immunology* **92**, 111–1117.
50. Hu, W. G., Chen, J., Battey, J. F., and Gu, X. X., (2000) Enhancement of clearance of bacteria from murine lungs by immunization with detoxified lipooligosaccharide from *Moraxella catarrhalis* conjugated to proteins. *Infect. Immun.* **68**, 4980–4985.

Immunoprotective Activity of CpG Oligonucleotides

Dennis M. Klinman, Ken J. Ishii,
and Ihsan Gursel

1. INTRODUCTION

Pathogens are a major source of morbidity and mortality worldwide. Despite the success of vaccines, antibiotics, and antiviral agents, the need persists to develop more effective means of combating pathogenic microorganisms. Boosting the activity of the innate immune system provides one method of protecting the host against a variety of infectious agents. The innate immune system is called upon physiologically to limit the early spread of pathogens *in vivo* (1,2). Host recognition of conserved determinants expressed by a diverse range of microorganisms, but absent from the host (such as lipopoly-saccharide (LPS), mannans or teichoic acid), stimulate the innate immune system to release immunoprotective cytokines, chemokines, and polyreactive antibodies (1–6). Without an adequate innate response, infections can become overwhelming and kill the host before antigen-specific immunity develops.

Recent studies indicate that, DNA of bacterial origin activates the innate immune system (7–10). Bacterial DNA contains approx 20-fold more unmethylated cytidine-phosphate Quanosine dinucleotide (CpG) motifs than mammalian DNA, owing to a combination of CpG suppression and CpG methylation (11,12). These unmethylated CpG motifs account for bacterial DNA's ability to stimulate an innate immune response (9,10,13–15).

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs mimic the immunostimulatory properties of bacterial DNA. CpG ODN enter immune cells within seconds, upregulate cytokine mRNA within minutes, and stimulate cytokine and Immunoglobulin M (IgM) release

within hours (9,10,16). These motifs directly activate lymphocytes, natural killer (NK) cells and macrophages (7,10,16,17). The chemokines elicited by CpG ODN promote immune cell migration and development of an inflammatory response (6). The cytokines elicited by CpG ODN include IL-12 and IFN γ , which promote the development of type 1 dependent cytotoxic T-cell responses, and IL-6, which promotes B-cell activation and antibody secretion (9,10). This array of cytokines has been shown to limit the early spread of intracellular pathogens (such as *Francisella tularensis*, *Listeria monocytogenes*, and *Salmonella typhimurium*) (18–20). Recognition of unmethylated CpG motifs has been evolutionarily conserved in species as diverse as fish and primates. This raises the possibility that activation of the innate immune system by CpG motifs may confer a selective advantage to the host (9). This review examines evidence that CpG induced immune responses protect the host from infectious pathogens, speed recovery from existing infections, and/or promote the development of long-lasting pathogen-specific immunity.

2. RESULTS

2.1. Immunoprotective Activity of CpG ODN

Zimmermann et al. were the first to document that CpG ODN could increase host resistance to infection (21). They studied the susceptibility of BALB/c mice to *Leishmania*. This strain of mice typically mounts a permissive Th2 rather than sterilizing Th1 response to *Leishmania* challenge, such that the parasite load increases by $>10^4$ within a few weeks of infection (22). When BALB/c mice were pretreated with CpG ODN and then challenged with *Leishmania*, the infecting parasites were eliminated (21). A significant reduction in parasite load was also observed when CpG ODN was administered up to 20 d after infection, indicating the value of therapeutic, as well as prophylactic intervention (21). Protection was attributed to CpG ODN-driven production of Th1-associated cytokines (primarily IL-12 and IFN γ) by professional antigen presenting cell (APC) (22).

Similar results have now been reported for other pathogens. Krieg et al. injected normal mice with 30 μ g of CpG ODN and challenged them up to 14 d later with 10 lethal doses (LD $_{50}$) of *Listeria*. Three days into the infection, the bacterial load in the livers and spleens of ODN treated animals was >100 -fold lower than untreated controls (23). Extending those findings, Elkins et al. demonstrated that multiple strains of normal mice survived infection by 10^3 – 10^5 LD $_{50}$ of *Listeria* or *Francisella* when pretreated with CpG ODN (Table 1) (24,25). Throughout these studies, treatment with ODN from which the critical CpG motif was eliminated by inversion or methylation failed to promote survival, demonstrating a key role for CpG induced immunity.

Table 1
Impact of CpG ODN on Survival from 10^3 LD₅₀ of *Listeria*

Treatment	Number of treatments	Day of challenge post Rx	% Survival
Bacterial DNA	1	3	100
Mammalian DNA	1	3	0
CpG ODN	1	3	100
GpC ODN*	1	3	0
C ^{meth} pG ODN*	1	3	0
PBS	1	3	0
CpG ODN	1	1	0
CpG ODN	1	2	60
CpG ODN	1	3	100
CpG ODN	1	10	90
CpG ODN	1	21	0
CpG ODN	20	2	100
CpG ODN	20	13	100
CpG ODN	20	21	30
CpG ODN	20	28	0
GpC ODN*	20	1	0
GpC ODN*	20	14	0

BALB/c mice were injected ip every 2 wk for up to 4 mo with 50 µg of ODN. They were challenged with 10^3 LD₅₀ of *L. monocytogenes* on the day shown after the last treatment. Data represent the percentage of mice surviving for >3 wk. Each group shown consists of 5–15 animals. All cases of 90–100% survival are statistically significant ($p < .05$).

*Note that these are control ODN, in which the CpG motif has been eliminated by inversion or methylation.

It should be appreciated that the proinflammatory and Th1 responses induced by CpG ODN do not protect against all pathogens: Yamamoto et al. reported that ODN failed to improve survival in mice challenged with influenza (26), and we were unable to protect against *Yersinia enterocolitica*, respiratory syncytial virus, or herpes simplex virus. It remains to be seen whether these limitations can be overcome by improving the half-life, activity, or cell specificity of CpG ODN.

2.2 Duration of Protective Immunity

The antigen-specific immune response induced by conventional vaccines develops over a period of weeks and persists for the life of the host. In contrast, the innate immune response elicited by CpG ODN takes several days to reach optimal activity, and provides protection for approx 2 wk (24,25).

Whereas CpG therapy successfully eliminates established infections involving slow growing pathogens (such as *Leishmania*) (21), protection against high-dose challenge by a rapidly proliferating microorganism requires that the innate immune system be preactivated. Thus, mice treated with CpG ODN and then immediately challenged with 10^3 LD₅₀ of *L. monocytogenes* do not survive, because the innate immune system needs a “head start” to mount an effective response against this pathogen (Table 1). Administering CpG 2 d prior to challenge results in 60% survival, whereas 100% survival results when mice are treated from 3–14 d prior to challenge (Table 1). The delay between ODN administration and the development of maximal protection suggests that a cascade of events, involving either

1. The activation/migration of immunoprotective cells.
2. The accumulation of humoral factors, contributes to this process.

Of interest, other responses mediated by CpG ODN, (such as their anti-allergic effects) also require several days for optimal effect (27). The success of ODN therapy thus reflects, the balance between how rapidly the innate immune system is activated vs the dose of pathogen, and its rate of proliferation.

2.3. Prolongation of Protective Immunity in vivo

The 2 wk of protective immunity provided by a single dose of CpG ODN, has a limited number of therapeutic applications, such as treating patients in the perioperative period, or improving the resistance of military personnel to infection prior to entry into high risk environments. Additional benefits would accrue if the duration of CpG induced protection were extended. Toward that end, we treated mice with CpG ODN every 2 wk for several months. A persistently heightened state of immune reactivity was maintained by this regimen (28). Specifically, serum IgM levels rose, and there were greater numbers of cells actively secreting IL-6 and IFN γ in vivo in repeatedly treated vs control mice. Moreover, animals injected every 2 wk for four months and challenged throughout that period with 10^3 LD₅₀ of *Listeria monocytogenes* or *Francisella tularensis* continued to resist infection (28).

2.4. CpG DNA Facilitates the Induction of Long-Term Pathogen-Specific Immunity

CpG ODN can be used as immune adjuvants, boosting the antigen-specific response elicited by conventional and DNA-based vaccines (15,29–31). This adjuvant effect suggests that animals treated with CpG ODN, and then exposing to a pathogen, might develop improved pathogen-specific immunity. To study this hypothesis, BALB/c mice were treated with CpG ODN and challenged to 3 d later with *L. monocytogenes*. As expected, all animals

Table 2
CpG ODN Promote Development of Pathogen-Specific Immunity

Rx	Challenge	Rechallenge	# Surviving/ # Challenged	% Survival
PBS	<i>Listeria</i>		0/10	0
	<i>Francisella</i>		1/10	10
CpG ODN	<i>Listeria</i>		20/20	100
	<i>Francisella</i>		10/10	100
CpG ODN	<i>Listeria</i>	<i>Listeria</i>	10/10	100
	<i>Listeria</i>	<i>Francisella</i>	0/10	0
CpG ODN		<i>Listeria</i>	0/10	0
		<i>Francisella</i>	0/10	0

BALB/c mice were injected ip with PBS or 50 µg of CpG ODN. Approximately 3 days later, animals were challenged with 10³ LD₅₀ of *L. monocytogenes* or *F. tularensis*. Mice that survived *L. monocytogenes* challenge were then rechallenged 45 d later. A control group was challenged only once, 45 d after initial treatment with CpG ODN.

Data show the percentage of mice surviving > 3 wk postchallenge.

survived (due CpG up-regulation of the innate immune system, Table 2). More than 1 mo later (after the protective effects of the ODN had worn off), the same animals were rechallenged with 10⁴ LD₅₀ of *L. monocytogenes* or 10² LD₅₀ of *F. tularensis*. One hundred percent of the animals survived *L. monocytogenes* challenge, whereas none survived *F. tularensis* (Table 2). Thus, long-term pathogen-specific immunity was induced when a lethal challenge was rendered sublethal by CpG ODN treatment.

2.5. Mechanism of Action of CpG ODN

Whereas 25–50 µg of CpG ODN protects BALB/c mice from a variety of infectious agents, this dose of ODN has little impact on the number of cells actively secreting Ig or cytokine in vivo (25,28). However, when spleen cells from ODN-treated mice were exposed to bacteria, they responded more rapidly and in greater number than cells from normal mice (25,28). These findings suggest that CpG ODN lower the activation threshold of cells in the innate immune system. To test this hypothesis, BALB/c mice were treated with CpG ODN or phosphate buffered saline (PBS), and then challenged 3 d later with 10³ LD₅₀ of *L. monocytogenes*. As seen in Table 3, the number of cells actively secreting IL-6 was significantly greater in ODN treated mice 12 h after *Listeria* infection than in challenged PBS treated controls (*p* <.01). This increase was not due to the proliferation of immune cells, since that requires >12 h. Moreover, ODN treatment was not associ-

Table 3
CpG ODN Accelerate Activation of Cytokine Secreting Cells Following Pathogen Exposure

CpG ODN <i>Treatment</i>	Hours post <i>Listeria</i> challenge	Fold increase in cytokine secreting cells	
		IL-6	IFN γ
PBS	12	1.3 $\pm$ 0.2	1.1 $\pm$ 0.1
CpG ODN	12	8.4 $\pm$ 1.3*	1.2 $\pm$ 0.1
PBS	72	2.6 $\pm$ 0.4	1.8 $\pm$ 0.8
CpG ODN	72	5.7 $\pm$ 0.7*	6.5 $\pm$ 1.2*

BALB/c mice were injected ip with 50 μ g of CpG ODN or PBS. These animals were challenged three days later with 10³ LD₅₀ of *L. monocytogenes*. The level of immune activation in vivo was monitored by ELISpot analysis 12 and 72 h after challenge.

Data represent the mean $\pm$ SD of six individually tested mice/group from two different experiments.

*Statistically significant increase in cytokine producing cell number in CpG ODN treated vs control mice ($p < .01$).

ated with an immediate increase in spleen cellularity. Three days after infection, there were significantly more cells secreting both IL-6 ($p < .01$) and IFN γ ($p < .01$) in CpG ODN than PBS treated mice (Table 3). These findings suggest that CpG ODN enabled the innate immune system to mount a more vigorous immune response immediately after challenge. Different cell types may contribute to this response over time, as shown by the rise in IFN γ secreting cell numbers at 3 d.

2.6. Characteristics of Immunoprotective CpG ODN

CpG ODN ranging from 10–40 bp in length are typically used in protection experiments. Such ODN are water soluble and are rapidly distributed in vivo after IM, IP, IV or ID administration. Indeed, recent studies show that 10–50 μ g of CpG ODN administered by any of these routes can protect mice from 10²–10³ LD₅₀ of a variety of bacterial or parasitic pathogens (18–21,23–25).

Several characteristics of CpG ODN make them attractive for clinical use. First, a single dose provides protection for approx 2 wk. Thus, ODN can be administered to populations “at risk” prior to pathogen exposure (25). Second, CpG ODN are effective against a wide range of pathogens, and thus may be of use before the causative agent is identified (e.g., exposure to biowarfare agents). Third, CpG ODN can improve the immune response of even immuno-suppressed individuals (including newborns, the elderly, and HIV patients) and thus can provide broad, population-based protection from infection (32). Finally, phosphorothioate modified CpG ODN (which are

commonly used in protection experiments) are readily manufactured at low cost and high purity, are stable in powder form at room temperature, and are easily reconstituted in sterile saline for rapid administration. Ongoing efforts to increase the half-life of CpG ODN in vivo and to identify motifs of optimal length and flanking sequence should further improve their therapeutic utility.

3. DISCUSSION

CpG ODN protect normal mice from infection by a variety of viral, bacterial, and parasitic microorganisms. A single dose of ODN confers protection for approximately two weeks, and persistent protection can be achieved by readministering these agents every two weeks. CpG ODN function by lowering the threshold for stimulation of cellular elements of the innate immune system, thereby improving the host's ability to respond quickly and effectively to pathogen challenge. The adjuvant effect of CpG ODN also facilitates the development of long-lasting antigen specific immunity following pathogen exposure.

The potential uses of CpG ODN are not limited to humans. In husbandry animals, serious infectious diseases are sometimes "treated" by slaughtering entire herds. For such diseases, infection is monitored by antibody screening, precluding the use of vaccines for disease control. Yet CpG ODN could prevent the spread of infection to neighboring herds without interfering with such surveillance.

CpG ODN can also be combined with other agents for use in postexposure treatment/vaccination. In such a situation, the innate immune response triggered by the ODN would suppress the proliferation of the pathogen, while promoting the host's ability to mount an antigen-specific response.

REFERENCES

1. Marrack, P. and Kappler, J. (1994) Submersion of the immune system by pathogens. *Cell* **76**, 323–332.
2. Medzhitov, R. and Janeway, C.A. (1998) Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295–298.
3. Medzhitov, R. and Janeway, C. A. (1997) Innate immunity: impact on the adaptive immune response. *Cur. Op. Immunol.* **9**, 4–9.
4. Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999) Maturation, activation and protection of dendritic cells induced by double stranded RNA. *J. Exp. Med.* **189**, 821–829.
5. Portnoy, D. A. (1992) Innate immunity to a facultative intracellular bacterial pathogen. *Cur. Op. Immunol.* **4**, 20–28.
6. Takeshita, S., Takeshita, F., Haddad, D. E., Ishii, K. J., and Klinman, D. M. (2000) CpG oligodeoxynucleotides induce murine macrophages to up-regulate chemokine mRNA expression. *Cell Immunology* **206**, 101–106.

7. Yamamoto, S., Yamamoto, T., Katoaka, T., Kuramoto, E., Yano, O. and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076.
8. Yamamoto, S., Yamamoto, T., Shimada, S., et al. (1992) DNA from bacteria, but not vertebrates, induces interferons, activate NK cells and inhibits tumor growth. *Microbiol. Immunol.* **36**, 983–997.
9. Krieg, A. M., Yi, A., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–548.
10. Klinman, D. M., Yi, A., Beaucage, S.L., Conover, J., and Krieg, A. M. (1996) CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN γ . *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
11. Bird, A. P. (1987) CpG-rich islands and the function of DNA methylation. *Trends Genet* **3**, 342–347.
12. Razin, A. and Friedman, J. (1981) DNA methylation and its possible biological roles. *Prog. Nucleic Acid Res. Mol. Biol.* **25**, 33–52.
13. Halpern, M. D., Kurlander, R. J. and Pisetsky, D. S. (1996) Bacterial DNA induces murine interferon-gamma production by stimulation of IL-12 and tumor necrosis factor-alpha. *Cell. Immunol.* **167**, 72–78.
14. Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S., Wagner, H., and Heeg, K. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
15. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1 promoting adjuvants. *Nature Medicine* **3**, 849–854.
16. Yi, A., Klinman, D. M., Martin, T. L., Matson, S., and Krieg, A. M. (1996) Rapid immune activation by CpG motifs in bacterial DNA. *J. Immunol.* **157**, 5394–5402.
17. Stacey, K. J., Sweet, M. J. and Hume, D. A. (1996.) Macrophages ingest and are activated by bacterial DNA. *J. Immunol.* **157**, 2116–2120.
18. Culkin, S. J., Rhinehart-Jones, T., and Elkins, K. L. (1997) A novel role for B cells in early protective immunity to an intracellular pathogen, *Francisella tularensis* strain LVS. *J. Immunol.* **158**, 3277–3284.
19. Elkins, K. L., Rhinehart-Jones, T. R., Culkin, S. J., Yee, D., and Winegar, R. K. (1997) Minimal requirements for murine resistance to infection with *Francisella tularensis* LVS. *Infect. Immun.* **64**, 3288–3293.
20. Izzo, A. A. and North, R. J. (1997) Evidence for an alpha/beta T cell-independent mechanism of resistance to mycobacteria. *J. Exp. Med.* **176**, 581–586.
21. Zimmermann, S., Egeter, O., Hausmann, S., et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine Leishmaniasis. *J. Immunol.* **160**, 3627–3630.
22. Heinzl, F. P., Sadick, M. D., Joladay, B. J., Coffman, R.L., and Locksley, R. M. (1989) Reciprocal expression of IFN γ or IL-4 during the resolution or progression of murine leishmaniasis. *J. Exp. Med.* **169**, 59–72.

23. Krieg, A. M., Homan, L. L., Yi, A. K., and Harty, J. T. (1998) CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* **161**, 2428–2434.
24. Klinman, D. M. (1998) Therapeutic applications of CpG-containing oligodeoxynucleotides. *Antisense and Nuc. Acid Drug Dev.* **8**, 181–184.
25. Elkins, K. L., Rhinehart-Jones, T. R., Stibitz, S., Conover, J. S., and Klinman, D. M. (1999) Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* **162**, 2291–2298.
26. Yamamoto, S., Yamamoto, T. and Tokunaga, T. (2000) Oligodeoxyribonucleotides with 5'-ACGT-3' or 5'-TCGA-3' sequence induce production of interferons. *Curr. Top. Microbiol. Immunol.* **247**, 23–40.
27. Sur, S., Wild, J. S., Choudhury, B. K., Alam, R., Sur, N., and Klinman, D. M. (1999) Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* **162**, 6284–6291.
28. Klinman, D. M., Conover, J. and Coban, C. (1999) Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect. Immun.* **67**, 5658–5663.
29. Klinman, D. M., Barnhart, K. M., and Conover, J. (1999) CpG motifs as immune adjuvants. *Vaccine* **17**, 19–25.
30. Sato, Y., Roman, M., Tighe, H., et al. (1996.) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
31. Klinman, D. M., Yamshchikov, G., and Ishigatsubo, Y. (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* **158**, 3635–3642.
32. Klinman, D. M., Conover, J., Bloom, E. T., and Weiss, W. (1998) Immunogenicity and efficacy of DNA vaccination in aged mice. *J. Gerontol.* **53A**, B282.

Protective Immunity of Immunostimulatory Sequences Against Mycobacterial Infection

Tomoko Hayashi, Christine H. Tran, Lucinda Beck,
Savita Rao, and Antonino Catanzaro

1. INTRODUCTION

1.1. *Mycobacterium avium* (*M. avium*) Infection

M. avium infection is a common opportunistic infection in patients infected with human immunodeficiency virus (AIDS; Acquired Immunodeficiency disease syndrome) (1,2), and can also cause disease in immunocompetent individuals. In AIDS, disseminated *M. avium* infection is a major cause of morbidity and mortality, and is extremely difficult to treat in these patients because it responds poorly to available antimycobacterial therapies (3).

M. avium predominantly infects and multiplies within macrophages (4) *M. avium* is known to attach to and enter macrophages with the help of complement, transferrin, and integrin receptors expressed on the surface of macrophages (5,6). Macrophages are activated by several cytokines, such as tumor necrosis factor (TNF)- α , granulocyte colony stimulating factor (G-CSF), and IFN- γ that stimulate macrophages to inhibit intracellular growth of *M. avium* (7). AIDS patients with low CD4⁺ counts are very susceptible to *M. avium* infection (8), suggesting that the CD4⁺ T-cell population is important in the resistance of *M. avium* infection, whereas CD8⁺ T cells do not appear to influence the resistance to *M. avium* infection (9).

1.2. Immunostimulatory Sequences and its Analogs (ISS-ODN) and Infection

Recent studies have demonstrated the ability of ISS-ODN to facilitate control of the intracellular pathogens, *Listeria monocytogenes*, (*L. monocytogenes*) *Leishmania major* (*L. major*), and *Francisella tularensis* (10,11,12). Stud-

ies with *L. monocytogenes* showed that injection of mice with *Escherichia coli* DNA or ISS-ODN resulted in a much lower bacterial burden in the spleen and liver (Krieg et al., 1998). Similarly, ISS-ODN conferred a protective immunity against primary infection and resistance to secondary infection with *L. major* (12). This antimicrobial activity was attributed mainly to ISS-ODN-induced release of cytokines such as IL-12 and to the subsequent induction of a protective Th1 and/or CTL response.

2. RESULTS AND DISCUSSION

2.1. Treatment with ISS-ODN Inhibits the Growth of *M. avium* in vitro

To examine whether treatment with ISS-ODN prior to infection can stimulate macrophages to inhibit the growth of *M. avium*, murine bone marrow-derived macrophages (mBMDM) were first treated with ISS-ODN or mutated (M)-ODN (3, 10, or 30 $\mu\text{g}/\text{mouse}$) for 72 h and then infected with *M. avium*. Whereas treatment of mBMDM with ISS-ODN prior to infection did not affect the attachment of *M. avium*, or its invasion into mBMDM, by day 7, treatment with ISS-ODN did inhibit intracellular growth of *M. avium* in mBMDM by 80% (Fig. 1, A-a). No significant difference in *M. avium* growth was seen among the different concentrations of ISS-ODN tested.

To evaluate the therapeutic antimycobacterial effect of ISS-ODN, infected mBMDM were treated with ISS-ODN for 7 d after infection, starting two hours after the time of infection. Treatment with a single dose of ISS-ODN significantly decreased the intracellular growth of *M. avium* in mBMDM by 68%, compared to CFU in infected cells treated with M-ODN or medium alone (Fig. 1, A-b).

2.2. ISS-ODN Transiently Inhibits the Growth of *M. avium* in vivo

To determine whether ISS-ODN can enhance resistance to *M. avium* infection *in vivo*, mice were treated with ISS-ODN and infected with *M. avium* three days later. At wk 2, the lungs of *M. avium*-infected mice treated with ISS-ODN prior to infection contained a significantly lower number of viable bacteria compared to control phosphate-buffered saline (PBS)-treated mice (Fig. 1, B-a). In addition, mice treated with ISS-ODN prior to *M. avium* infection had nearly two log less bacteria in the spleen at four weeks compared to the (PBS)-treated mice (Fig. 1, B-b). By 6 wk, however, splenic CFU was similar in control and ISS-ODN groups. There was no significant difference in the mycobacterial loads in the liver of mice among the treatment groups at any of these time points (Fig. 1, B-c). Thus, a single injection

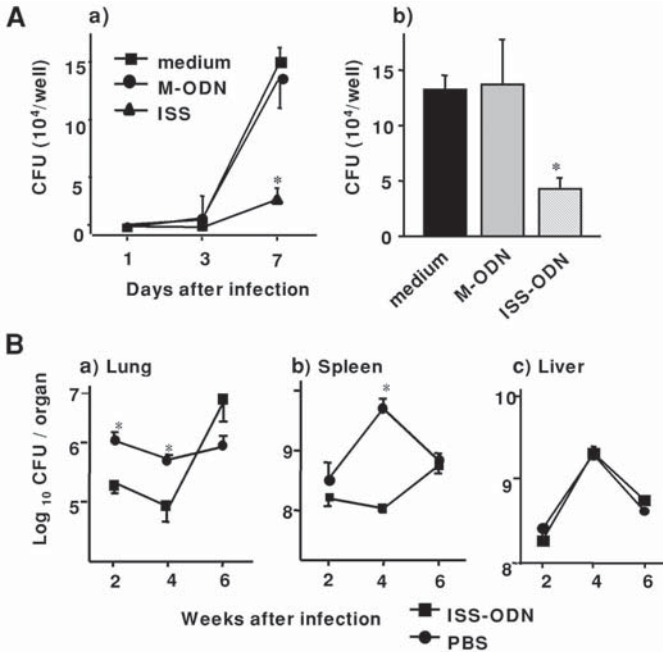


Fig. 1. (A) Antimycobacterial effects of ISS-ODN in vitro. (a) mBMDM from C57Bl/6 mice were adhered on 96-well plates and treated with ISS-ODN (5'-TGACTGTGAACGTTTCGAGATGA-3') for three days prior to infection. Macrophages treated with M-ODN (5'-TGACTGTGAACCTTAGAGATGA-3') or media alone served as controls. mBMDM were then infected with *M. avium* [strain I13 isolated from AIDS patients (20)] at a cell:bacteria ratio of 1:0.5 and intracellular growth of *M. avium* was assessed by the CFU (colony forming unit) assay on days 1, 3, and 7 after infection. (b) Adherent mBMDM were first infected with *M. avium* for 2 h. The infected cells were cultured with fresh media containing ISS-ODN or M-ODN immediately after infection. *M. avium* growth was assessed by the CFU assay 7 days postinfection. Each condition was tested in triplicate and the results were expressed as mean $\pm$ SD of CFU per well. Results shown are representative of three experiments. * $p < 0.01$ compared to CFU recovered from cells treated with M-ODN or medium alone. (B) Antimycobacterial effects of ISS-ODN in vivo. C57Bl/6 mice ($n = 5$ per treatment group) were injected with ISS-ODN (50 μ g/mouse) or PBS intradermally three days before infection with 10^7 *M. avium* per mouse. The control mice received PBS. At 2, 4, and 6 wk after infection, mice were sacrificed and the spleen, liver, and lungs from each mouse were collected and weighed. *M. avium* growth in the lungs (a), spleen (b), and liver (c) of the animals was assessed by the CFU assay. Results shown are mean $\pm$ SD of the number of CFU per organ. * $p < 0.05$ compared to CFU in the organs of control mice that received PBS instead of ISS-ODN.

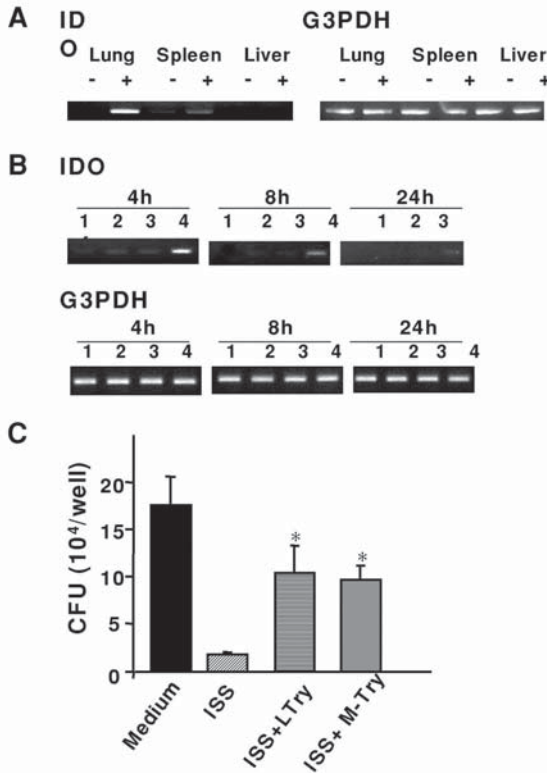


Fig. 2. IDO is involved in the antimycobacterial effect of ISS-ODN. **(A)** ISS-ODN induced IDO gene expression in the lungs and spleen, but not liver. Uninfected mice were injected with ISS-ODN 50 μ g/mouse) and the lungs and spleen were collected 16 h after injection. Total RNA was isolated and semi-quantitative RT-PCR was performed to assess the induction of IDO gene expression. The primer sequences used were as follows: IDO: 5'-TTATGCAGACTGTGTCCTGGCAAA-3', and 5'-TT TCCAGCCAGACA GATATATGCG-3', G3PDH: 5'-ACCACAGTCCATGCCATCAC-3', and 5'-TCCACCACCCTGTTGCTGTA-3'. PCR products were visualized by electrophoresis on 2% Agarose gels. -, injected with saline; +, injected with ISS-ODN. **(B)** *M. avium* infection with ISS-ODN treatment of mBMDM induces IDO gene expression. mBMDM were treated with ISS-ODN for three days prior to infection with *M. avium*. Total RNA was isolated from mBMDM and evaluated by semi-quantitative RT-PCR to assess the induction of IDO gene expression at 4, 8, and 24 h postinfection. 1. Medium alone; 2. ISS-ODN treatment alone; 3. *M. avium* infection alone; 4. ISS-ODN treatment and *M. avium* infection. **(C)** Addition of excess L-tryptophan (L-try) or competitive IDO inhibitor l-methyl-DL-tryptophan (M-try) partially inhibited the anti-mycobacterial effect of ISS-ODN. mBMDM were treated with ISS-ODN for 3 d in the presence of excess L-try (final concentration of 66 μ g/mL) or M-try (125 μ M) and subsequently infected with *M. avium* for

of ISS-ODN significantly reduced the mycobacterial growth in the spleen and lungs, but not in the liver. This protective effect was transient and most apparent at two and 4 wk after a single administration of ISS-ODN.

Although treatment with ISS-ODN after infection inhibited *M. avium* growth in mBMDM *in vitro*, there was no difference in the mycobacterial counts in the spleen or liver at any observed time point when we used ISS-ODN 1 wk after infection *in vivo*. Overall, ISS-ODN significantly reduced the growth of *M. avium* when used in a preventive mode, but not when used in a therapeutic mode.

2.2.1. ISS-ODN Protection *in vivo* is not Mediated Through Augmentation of the T-cell Response

The observation that ISS-ODN protects isolated macrophages *in vitro* (Fig. 1 A) and that the protective effect observed in ISS-ODN-treated mice is transient (Fig. 1 B) suggests that the antimycobacterial effect of ISS-ODN is mediated by a T-cell independent mechanism of protection via innate immunity. To further investigate the potential role of adaptive immunity in this model of ISS-ODN-mediated protection against *M. avium*, mice were treated with ISS-ODN or M-ODN, infected with *M. avium*, and then their T-cell response was evaluated. The mice were sacrificed at 3 wk postinfection, and the splenocytes were restimulated with anti-CD3 and anti-CD28 antibodies to amplify the response from preexisting memory and activated T cells. FACS-based intracellular cytokine staining was used to determine the frequencies of IFN- γ producing CD4⁺ T cells. There was a dramatic increase in the IFN- γ response of the CD4⁺ T cells in the infected vs uninfected mice, demonstrating that the observed Th1 response is infection-specific. However, treatment of *M. avium*-infected mice with ISS-ODN did not further increase the frequency of IFN- γ positive CD4⁺ T cells (data not shown). These data suggest that it is unlikely T-cell dependent immunity plays a role in the antimycobacterial effect of ISS-ODN during early stages of infection.

2.3. ISS-ODN Inhibition of *M. avium* growth in Macrophages is Independent of iNOS, NADPH Oxidase, IL-12, TNF- α , IFN- α/β , and IFN- γ

To further investigate the mechanisms of the antimycobacterial effects of ISS-ODN, mice with targeted disruptions of genes known to play roles in *M. avium* infection were used. mBMDM from NADPH oxidase^{-/-}, iNOS^{-/-}

seven days in the presence of L-try or M-try. Intracellular growth of *M. avium* was assessed by the CFU assay. Each condition was tested in triplicate and the results expressed as mean $\pm$ SD of CFU per well. Results shown are representative of three experiments. * $p < 0.01$ compared to CFU recovered from cells treated with ISS-ODN.

TNF- $\alpha^{-/-}$, IL-12p40 $^{-/-}$, IFN- α/β R $^{-/-}$ and IFN- γ R $^{-/-}$ mice were treated with ISS-ODN for 3 d, and then infected with *M. avium*. By 7 d after infection, ISS-ODN inhibited the intracellular growth of *M. avium* in mBMDM from these knockout mice by 60–85%, similar to wild-type mice (data not shown). These results indicate that these gene products (e.g., nitrogen intermediates, oxygen radicals TNF- α and so on) are not central to the antimycobacterial effect induced by ISS-ODN in vitro.

2.4. Induction of Indoleamine 2,3-Dioxygenase (IDO) Contributes to the Antimycobacterial Activity of ISS-ODN

Macrophages employ effector mechanisms that deny the pathogens substrates required for their growth. One such example is the depletion of tryptophan owing to the induction of IDO. IDO is induced by IFNs (13) and is the rate-limiting enzyme in the catabolism of tryptophan. Induction of this enzyme limits the availability of this important amino acid to invading pathogens, such as *Toxoplasma gondii* (14), *Plasmodium berghe* in a murine model of malaria (15), *Chlamydia psittaci* (16), and *Chlamydia trachomatis* (17).

In vivo, IDO gene induction was observed in the lungs and spleen, but not in the liver, 16 h after injection of ISS-ODN (Fig. 2, A). This organ-specific induction of IDO induced by ISS-ODN agrees with the observed pattern of *M. avium* inhibition (Fig. 1, B). In in vitro studies, optimal induction of IDO gene transcription required both treatment with ISS-ODN and infection with *M. avium* (Fig. 2, B).

In order to investigate the role of IDO in the inhibition of *M. avium* growth, mBMDM were cultured with the IDO competitive inhibitor 1-methyl-DL-tryptophan (M-try) or with excess L-tryptophan (L-try). When the *M. avium*-infected macrophages were cultured with ISS-ODN in the presence of L-try or M-try, four-fold reductions in the antimycobacterial ability of ISS-ODN treatment were observed (Fig. 2, C). Taken together, these data suggest that IDO plays a role in the observed anti-mycobacterial properties of ISS-ODN. Although mycobacteria are able to synthesize all amino acids required for their growth including tryptophan (18), the inhibition of *M. avium* growth is likely owing to the combined result of reduced tryptophan availability and increased levels of the toxic metabolites L-kynurenine, anthranilic acid, quinolinic acid, and picolinic acid (19).

2.5. ISS-ODN is an Adjunct to Antimycobacterial Therapy with CLA

Treatment of *M. avium* infection by conventional chemotherapy is difficult and requires prolonged administration (3). In in vivo experiments, ISS-ODN did not affect the colony counts when administered in an established

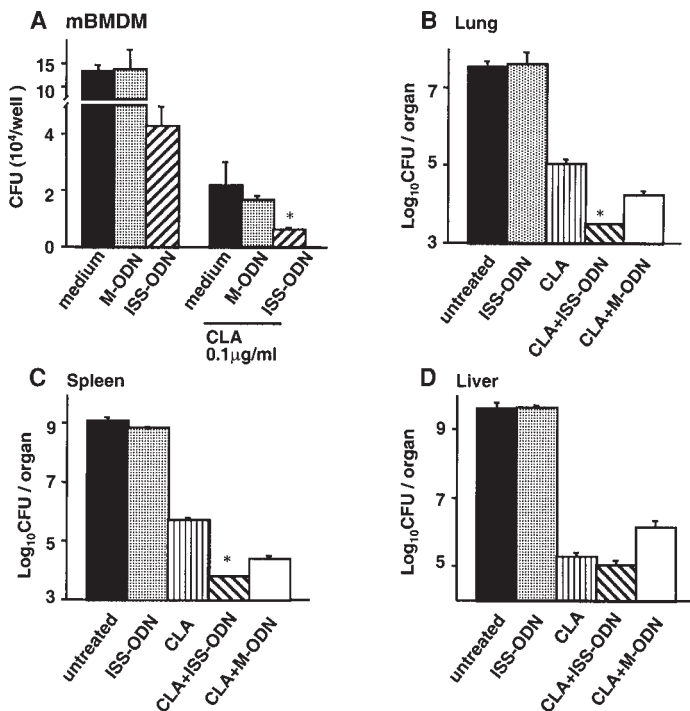


Fig. 3. ISS-ODN enhances the antimycobacterial effect of CLA. (A) Enhancement of the antimycobacterial effect of CLA by ISS-ODN in mBMDM in vitro. Infected macrophages were treated with ISS-ODN or M-ODN (50 μ g/mouse) in the presence or absence of CLA (0.1 μ g/mL) and the intracellular growth of *M. avium* was assessed by the CFU assay on d 7 after infection. Each condition was tested in triplicate and expressed as mean $\pm$ SD of CFU per well. Results shown are representative of three experiments. * p < 0.01 compared to CFU recovered from cells treated with medium alone. (B, C, and D) Enhancement of the antimycobacterial effect of CLA by ISS-ODN in mBMDM in vivo. C57Bl/6 mice were infected with 10^7 *M. avium* and then treated with ISS-ODN alone, CLA alone, CLA and ISS-ODN, and CLA and M-ODN (n = 5 per group). CLA was administered (200 mg/kg) intraperitoneally every other day three times a week for four weeks. *M. avium* growth in the lung (B), spleen (C), and liver (D) of the animals was determined by the CFU assay at eight weeks after infection. The data shown represent one experiment performed with similar results. Results shown are mean $\pm$ SD of the number of CFU per organ. * p < 0.05 compared to CFU in the organs of control mice that received CLA only, or a combination of CLA and M-ODN. Results shown are mean $\pm$ SD.

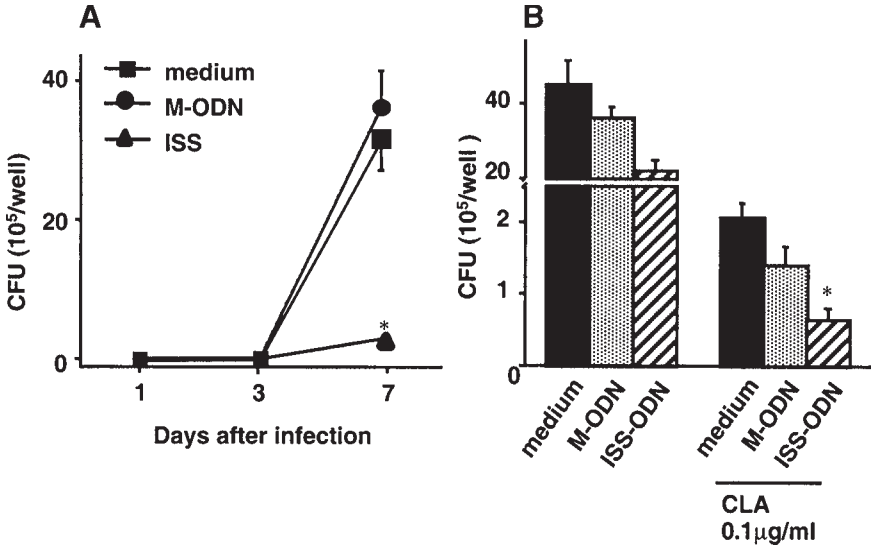


Fig. 4. ISS-ODN has antimycobacterial properties in human macrophages. **(A)** Human monocytes (hMMD) were treated with ISS-ODN or M-ODN (3 μ g/mL) for three days prior to *M. avium* infection and intracellular growth of *M. avium* by the CFU assay was assessed on d 1, 3, and 7 after infection. **(B)** hMMD were treated with ISS-ODN or M-ODN immediately after infection and *M. avium* growth was assessed by the CFU assay seven d postinfection. Each condition was tested in triplicate, and the results have been expressed as mean $\pm$ SD of CFU per well. Results shown are representative of three experiments. * $p < 0.01$ compared to CFU recovered from cells treated with M-ODN or medium alone.

infection. Therefore, we hypothesized that co-administration of ISS-ODN, along with a chemotherapeutic agent such as CLA, may further enhance mycobacterial clearance.

M. avium-infected mBMDM were treated with CLA in the presence or absence of ISS-ODN or M-ODN and *M. avium* growth in vitro was determined 7 d after infection. ISS-ODN and CLA, when used individually, reduced bacterial growth in mBMDM by 68 and 84%, respectively (Fig. 3 A). When ISS-ODN was used together with CLA, bacterial counts were further reduced (95%), compared to medium alone (Fig. 3 A).

In vivo, treatment with CLA alone decreased bacterial growth in the lungs (1.5 log reduction, Fig. 3 B), spleen (3.5 log reduction, Fig. 3 C), and liver (4 log reduction, Fig. 3 D) In this therapeutic model, ISS-ODN alone did not

inhibit the growth of *M. avium*. However, when ISS-ODN was combined with CLA, there was a further reduction of bacterial counts particularly in the lungs (3 log reduction, Fig. 3 C) and in the spleen (2 log reduction, Fig. 3 C). Despite the fact that treatment with ISS-ODN post-infection did not affect the course of the infection in vivo, these findings suggest that ISS-ODN can enhance the therapeutic efficacy of CLA in the setting of established *M. avium* infection.

2.6. ISS-ODN Inhibits *M. avium* Growth in Human Macrophages in vitro

To study the relevance of the antimycobacterial effects of ISS-ODN in humans, human monocyte-derived macrophages (hMDM) were treated with ISS-ODN or M-ODN and then infected with *M. avium*. There was maximal inhibition of *M. avium* growth at 3 µg/mL of ISS-ODN (Fig. 4 A) with no further increase in inhibition at the higher concentrations (data not shown). At seven days postinfection, treatment with ISS-ODN inhibited intracellular growth of *M. avium* by 91% (Fig. 4 A). In the study of the therapeutic effects of ISS-ODN on established *M. avium* infection, treatment with ISS-ODN significantly decreased the intracellular growth of *M. avium* in hMDM by 53% (Fig. 4 B). When infected cells were treated with ISS-ODN together with CLA, *M. avium* growth was further inhibited up to 99% ($p < 0.01$), compared to medium alone (Fig. 4 B). These findings indicate that ISS-ODN can provide a significant antimycobacterial effect in human macrophages as well as in the mouse model.

In summary, this study demonstrates that administration of ISS-ODN enhances resistance against *M. avium* infection through the induction of IDO. ISS-ODN itself provides limited protection against *M. avium*. However, this effect can be amplified upon co-delivery with an antimycobacterial drug. The combined administration of ISS-ODN with other antibiotics, or other antimicrobial agents, provides a novel therapeutic strategy for microbial infections and warrants further investigation.

ACKNOWLEDGMENTS

We thank Dr. E. Raz for his helpful comments. This work was supported in part by funds from the National Institutes of Health (grants AI40682 and AI47078) and from Dynavax Technologies Corporation.

REFERENCES

1. Horsburgh, C. R., Jr. (1991). *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **324**, 1332–1338.

2. Prince, D. S., Peterson, D. D., Steiner, R. M., et al. (1989) Infection with *Mycobacterium avium* complex in patients without predisposing conditions. *N. Engl. J. Med.* **321**, 863–868.
3. Masur, H. (1993) Recommendations on prophylaxis and therapy for disseminated *Mycobacterium avium* complex disease in patients infected with the human immunodeficiency virus. Public Health Service Task Force on Prophylaxis and Therapy for *Mycobacterium avium* Complex. *N. Engl. J. Med.* **329**, 898–904.
4. Edwards, D. and Kirkpatrick, C. H. (1986) The immunology of mycobacterial diseases. *Am. Rev. Respir. Dis.* **134**, 1062–1071.
5. Bermudez, L. E., Young, L. S., and Enkel, H. (1991) Interaction of *Mycobacterium avium* complex with human macrophages: roles of membrane receptors and serum proteins. *Infect. Immun.* **59**, 1697–702.
6. Rao, S. P., Ogata, K. and Catanzaro, A. (1993) *Mycobacterium avium*-M. intracellulare binds to the integrin receptor alpha v beta 3 on human monocytes and monocyte-derived macrophages. *Infect. Immun.* **61**, 663–670.
7. Sarmiento, A. and Appelberg, R. (1996) Involvement of reactive oxygen intermediates in tumor necrosis factor alpha-dependent bacteriostasis of *Mycobacterium avium*. *Infect. Immun.* **64**, 3224–3230.
8. Crowe, S. M., Carlin, J. B., Stewart, K. I., Lucas, C. R. and Hoy, J. F. (1991) Predictive value of CD4 lymphocyte numbers for the development of opportunistic infections and malignancies in HIV-infected persons. *J. Acquir. Immune Defic. Syndr.* **4**, 770–776.
9. Bermudez, L. E. & Petrofsky, M. (1999). Host defense against *Mycobacterium avium* does not have an absolute requirement for major histocompatibility complex class I-restricted T cells. *Infect. Immun.* **67**, 3108–3111.
10. Klinman, D. M., Conover, J. and Coban, C. (1999) Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect. Immun.* **67**(11), 5658–5663.
11. Krieg, A. M., Love-Homan, L., Yi, A. K. and Harty, J. T. (1998) CpG DNA induces sustained IL-12 expression in vivo and resistance to *Listeria monocytogenes* challenge. *J. Immunol.* **161**, 2428–2434.
12. Walker, P. S., Scharton-Kersten, T., Krieg, A. M., et al. (1999) Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-gamma-dependent mechanisms. *Proc. Natl. Acad. Sci. USA* **96**, 6970–6975.
13. Feng, G. S., Dai, W., Gupta, S. L., et al. (1991). Analysis of interferon-gamma resistant mutants that are possibly defective in their signaling mechanism. *Mol. Gen. Genet.* **230**, 91–96.
14. Pfefferkorn, E. R. and Guyre, P. M. (1984) Inhibition of growth of *Toxoplasma gondii* in cultured fibroblasts by human recombinant gamma interferon. *Infect. Immun.* **44**, 211–216.
15. Sanni, L. A., Thomas, S. R., Tattam, B. N., et al. (1998) Dramatic changes in oxidative tryptophan metabolism along the kynurenine pathway in experimental cerebral and noncerebral malaria. *Am. J. Pathol.* **152**, 611–619.

16. Carlin, J. M., Borden, E. C., and Byrne, G. I. (1989) Interferon-induced indoleamine 2,3-dioxygenase activity inhibits *Chlamydia psittaci* replication in human macrophages. *J. Interferon Res.* **9**, 329–337.
17. Beatty, W. L., Belanger, T. A., Desai, A. A., Morrison, R. P., and Byrne, G. I. (1994) Tryptophan depletion as a mechanism of gamma interferon-mediated chlamydial persistence. *Infect. Immun.* **62**, 3705–3711.
18. Ratledge, C. (1982) Nutrition, growth and metabolism. In *The biology of the Mycobacteria* (Ratledge, C. & Stanford, J., eds.), Vol. 1, pp. 183–271. 3 vols. Academic Press, London.
19. Pais, T. F. and Appelberg, R. (2000) Macrophage control of mycobacterial growth induced by picolinic acid is dependent on host cell apoptosis. *J. Immunol.* **164**, 389–397.
20. Cho, H. J., Takabayashi, K., Cheng, P. M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514.
21. Meylan, P. R., Richman, D. D. and Kornbluth, R. S. (1990) Characterization and growth in human macrophages of *Mycobacterium avium* complex strains isolated from the blood of patients with acquired immunodeficiency syndrome. *Infect. Immun.* **58**, 2564–2568.

PART VI
APPLICATIONS—ALLERGY

DNA-Based Immunotherapeutics For Allergic Disease

Anthony A. Horner and Eyal Raz

1. INTRODUCTION

Allergic diseases are a growing health concern in industrialized countries. Despite a number of effective therapeutic options for the prevention and treatment of the pathophysiologic responses that occur upon allergen exposure, the induction of true allergen desensitization remains an elusive therapeutic goal. Only immunotherapy (IT) has been shown to have any effect on the underlying hypersensitivities, which mediate allergic responses and traditional protein based allergen IT has a limited scope of efficacy. However, a number of reagents collectively termed DNA based immunotherapeutics have proven highly effective in both the prevention and reversal of Th2 mediated hypersensitivity states in mouse models of allergic disease. Four basic DNA based immunotherapeutic modalities have been used for these studies. These include immunization with gene vaccines, allergen mixed with immunostimulatory oligodeoxynucleotide (ISS-ODN), and physical allergen:ISS-ODN conjugates (AIC), as well as immunomodulation with ISS-ODN alone. This chapter will provide a framework for understanding the application of these DNA based immunotherapeutic strategies to the treatment of allergic disease.

2. PRESENT THERAPEUTIC OPTIONS FOR THE TREATMENT OF ALLERGIC DISEASE

Allergic diseases affect as many as 30% of individuals in some populations, and incidence and prevalence rates are continuing to rise (1–3). Ironically, these trends are occurring at a time when our understanding of the pathophysiologic mechanisms of atopy are fairly well developed. Further-

more, a large number of effective therapies are available for the treatment of many allergic diseases, such as corticosteroids for the reversal of allergic inflammation, antihistamines and leukotrienes for the attenuation of pathology associated with mast cell and eosinophil degranulation, and β_2 -receptor agonists for the reversal of bronchospasm (4,5). However, none of the pharmacological agents presently available effectively reverse the underlying allergic hypersensitivities, which perpetuate the allergic march. Allergen desensitization strategies have been developed for the reversal of allergic hypersensitivities. However, standard protein based IT,

1. Requires repeated subcutaneous (s.q.) injections,
2. Has an overall success rate of around 30–70% for respiratory allergies,
3. Has associated risks,
4. Is ineffective and unsafe for the treatment of food allergies,
5. Has debatable effectiveness for the treatment of asthma (6–8).

Thus, there is a clear need for more effective strategies for the prevention and reversal of the Th2 biased immune dysregulation, which fuels the pathogenesis of allergic conditions.

In contrast to the limitations of present therapies, a number of reagents collectively termed DNA based immunotherapeutics have proven highly effective in both the prevention and reversal of Th2 mediated hypersensitivity states in mouse models of allergic disease (9). Results from many laboratories have generated guarded optimism that DNA based immunotherapeutics may be effective for the reversal of allergic hypersensitivity states in humans and several clinical trials have already been initiated.

3. DNA-BASED VACCINATION FOR THE TREATMENT OF ALLERGIC DISEASE

Traditional allergen IT is based on the principles of vaccination against infectious agents. The therapeutic goal of IT in clinical practice is to elicit a protective immune response to an allergen, to which a clinical hypersensitivity preexists by effecting a change in the allergen specific adaptive immune repertoire. DNA-based vaccination schemes have shown great promise, when compared to the experimental equivalent of standard allergen IT, in rodent models of allergic disease. Three basic DNA-based vaccination strategies have been utilized for this purpose. Gene and protein/ISS-ODN vaccination have proven highly effective in the induction of Th1 and reversal of Th2 biased immune profiles (10–16). As a consequence of preventing and reversing underlying allergic sensitivities, these DNA-based vaccination strategies have been further shown to attenuate the immediate hypersensitivity response of anaphylaxis, and the late phase allergic response of asthma. Recently, physical conjugates of allergen and ISS-ODN (AIC)

have been developed. AIC have proven even more immunogenic than allergen gene expression plasmids and allergen/ISS-ODN cocktails in rodents and primates, and less allergenic than allergen extracts in *in vitro* studies with human basophils, and in skin testing studies of allergic patients (*see* Chapter 14) (17–20). These attributes suggest that AIC may be even safer and more effective than allergen expressing plasmids and allergen mixed with ISS-ODN for IT in allergic patients. The anti-allergic bioactivity of DNA based reagents has been well characterized within the context of the traditional vaccination paradigm, and the Th1 biased immune responses elicited have been shown to be long lasting, with antigen specific immunity being detectable up to one year or more after immunization (9).

4. DNA-BASED IMMUNOMODULATION FOR THE TREATMENT OF ALLERGIC DISEASE

Along with its use as an antiallergic vaccine adjuvant, an alternative paradigm for the application of ISS-ODN to the treatment of allergic disease has evolved. The concept is that of allergen independent immunomodulation. Preliminary investigations established that ISS-ODN was a potent Th1 adjuvant for antigens encountered at the site of delivery for up to 14 d (21, 22). Furthermore, ISS-ODN delivery up to seven d prior to primary exposure, prevented Th2 biased immune deviation when the antigen was delivered with a Th2 adjuvant (*i.e.* alum; *see* Chapter 13). Several laboratories have now established that previously sensitized mice treated with ISS-ODN in the absence of allergen have an attenuated hypersensitivity response to subsequent allergen challenge for a month or longer (23–26). In fact, ISS-ODN has proven to be as effective an immunomodulator as corticosteroids in the prevention of the immediate phase hypersensitivity response of allergic conjunctivitis, and more effective than corticosteroids in the prevention of the late phase inflammatory responses seen in murine allergic conjunctivitis and asthma models (*see* Chapters 23 and 25). Furthermore, like corticosteroids, allergen independent ISS-ODN delivery has been shown to inhibit allergen specific IL-5 production from Th2 sensitized mice. However, in contrast to corticosteroids, ISS-ODN immunomodulation also stimulates allergen specific IFN γ production from previously Th2 sensitized mice.

Recent *in vitro* human studies provide insight into the mechanisms by which ISS-ODN functions as an allergen independent antiallergic immunomodulator. These investigations have demonstrated that ISS-ODN elicits antigen independent production of Th1 promoting cytokines (*i.e.*, IL-12, IFN α , and IFN γ) and IL-10 from PBMC's, consistent with previous murine studies (27–30). In addition, ISS-ODN has been shown to increase IFN γ R (CD119), and reduce IL-4R (CD124) expression on human B cells (29).

Finally, it has been demonstrated that ISS-ODN inhibits IL-4 dependent IgE synthesis from human B cells in an IL-12, IFN α , IFN γ , and IL-10 dependent manner (28,29). These studies demonstrate that ISS-ODN inhibits allergic immune dysregulation by inducing the production of anti-allergic cytokines, and further suggest that ISS-ODN regulates cytokine receptor expression to favor responsiveness to IFN γ over IL-4. Along with providing a framework for understanding the intrinsic antiallergic immunomodulatory activities of ISS-ODN, these investigations further suggest that ISS-ODN- based immunomodulation may be effective in humans.

5. COMPARING DNA-BASED VACCINATION AND ISS-ODN IMMUNOMODULATION

In further consideration of the dichotomy between DNA-based vaccination and ISS-ODN-mediated immunomodulation, we have found that in contrast to the long standing and antigen-specific immune responses elicited with DNA-based vaccination, allergen nonspecific ISS-ODN, induced immunomodulation is relatively short-lived. In a series of studies, we have found that a single delivery of ISS-ODN shortly before a primary allergen challenge, leads to a shift in the allergen-specific cytokine response from a Th2 to a Th1 bias, and a dramatic inhibition of the late phase asthmatic response. These mice are protected from a second allergen challenge for an additional month. However, 2 mo after ISS-ODN delivery and primary allergen challenge, the antigen-specific immune and hypersensitivity responses to a second allergen challenge are similar to those of mice that did not receive ISS-ODN (24). Interestingly, Sur and colleagues demonstrated that two serial encounters with ISS-ODN, followed by allergen days later, leads to approx a 6-wk window of protection from a third allergen challenge, both in terms of the allergen-specific immune profile, which maintains the ISS-ODN-induced Th2 to Th1 cytokine bias, and the late phase asthmatic hypersensitivity response after challenge (26).

The principles of vaccination and immunomodulation, as discussed in this chapter, represent two distinct yet interrelated strategies for the utilization of DNA-based reagents in the treatment of allergic diseases (Fig. 1). With DNA-based vaccination, a long lasting Th1 biased adaptive immune response develops. This Th1 biased immune profile both prevents and reverses allergen specific Th2 biased T- and B-cell responses, and consequently, animals vaccinated with DNA-based vaccines are protected from allergic hypersensitivity responses for an extended period of time. In contrast, ISS-ODN induced immunomodulation appears to depend on the anti-allergic innate response that develops when immunocytes are exposed to

ISS-ODN. The authors' observations, in conjunction with those made by Broide, Sur and others, suggest that ISS-ODN-induced cytokine production and modulation of cytokine receptor expression (i.e., increased IFN γ R and decreased IL-4R expression) attenuates hypersensitivity responses in previously sensitized mice upon challenge, for a limited period of time (23–26, 29). It is likely that ISS-ODN-induced immunomodulation requires repeated exposures to allergen in the context of ISS-induced innate immune activation, to imprint a Th1 bias on the adaptive immune system. These repeated exposures to allergen and ISS-ODN would serve to fully vaccinate mice, and commit their antigen-specific lymphocytes to a Th1 phenotype. This schema for the interrelationship between the ISS-ODN effect on innate and adaptive immunity in the context of allergic disease is presented in Fig. 1.

6. A POTENTIAL ROLE FOR ENDOGENOUS IMMUNOSTIMULATORY DNA IN THE PREVENTION OF ATOPY

It has been proposed that the sterilization of the environment, and a reduction in the incidence of many infectious diseases may contribute to the rising incidence and prevalence rates of allergic diseases in industrialized countries (1,2), immunostimulatory DNA sequences are frequent in bacterial genomes and rare in mammalian DNA (9,21,30). Furthermore, ISS-ODN activates Toll like receptor-9 (TLR-9), and its signaling pathway in a manner analogous to signaling by other pathogen associated molecular patterns (PAMPs), which also induce immune activation by binding TLRs (*see* Chapter 4). Given that ISS-ODN-based interventions efficiently prevent the allergic phenotype, it is tempting to speculate that natural microbial flora in the intestines and microbes in inspired air activate mammalian immunity, to prevent the development of Th2 biased immune dysregulation via PAMP/TLR (i.e., PAMP/TLR-9) interactions. If the sterilization of the environment we live in is contributing to the rising health burden of allergic diseases presently felt in industrialized countries, as proposed by the “hygiene hypothesis,” then ISS-ODN based therapeutics may provide protection against the “allergic march,” without the inherent risks of natural infectious encounters. These concepts are discussed further in Chapter 23.

7. CONCLUSION

Over the past decade, a growing body of experimental evidence suggests that DNA-based immunotherapeutics may be effective in the treatment of allergic diseases, and the reversal of the allergen specific hypersensitivities that mediate them. DNA-based reagents have proven effective in the preven-

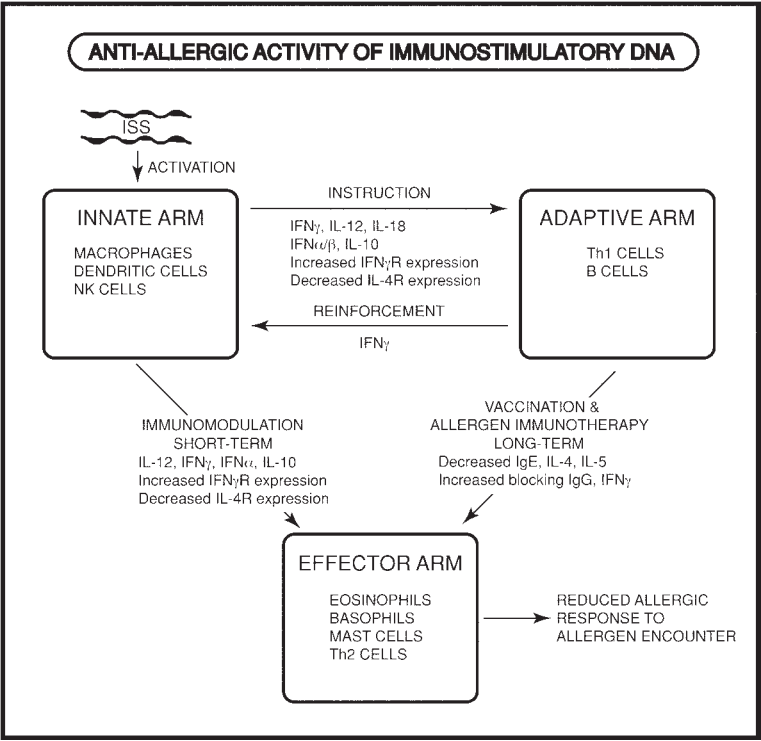


Fig. 1. Schema for the antiallergic activities of immunostimulatory DNA sequences (ISS). Cryptic immunostimulatory DNA contained in allergen gene vaccination plasmids and synthetic ISS-ODN, directly activate cells of the innate arm of the immune system to produce a number of antiallergic and Th1 promoting cytokines, while increasing the expression of IFN γ R and reducing the expression of IL-4R. This acute burst of cytokines, and potentially the modulation of cytokine receptor expression, directly inhibit the effector arm of the allergic hypersensitivity response, including the response of pre-committed Th2 cells. In addition, ISS-induced innate immune activation, promotes the development of Th1 and inhibits Th2 biased adaptive responses to allergens. This Th1 biased adaptive response reinforces the activities of innate immunocytes, while mediating long lasting inhibition of the effector arm of the allergic hypersensitivity response. Thus, ISS activation of two separate, but complementary arms of the immune response (innate and adaptive) has the potential to provide both rapid and long lasting inhibition of the allergic response.

tion and treatment of allergic diseases when used according to the principles of immunization. ISS-ODN has further proven to have utility as an immunomodulator when used independently of allergen, much in the way that corticosteroids are presently used to treat allergic diseases. Although their effects on immediate and late phase allergic hypersensitivity responses appear similar, DNA-based vaccination appears to have a much longer effect on adaptive immunity than ISS-ODN-induced immunomodulation. However, if utilized in clinical practice, ISS-ODN induced immunomodulation has a practical advantage over DNA-based vaccination, as it does not require that a patient's specific allergic hypersensitivities be identified. In the years to come, a number of clinical trials evaluating the safety and efficacy of DNA based immunotherapeutics for the treatment of allergic diseases are anticipated, and some are already underway. If these reagents prove as effective in humans as they have in rodents and nonhuman primates, then DNA-based immunotherapeutics are likely to revolutionize the standard of care for the treatment of allergic diseases.

REFERENCES

1. Holgate, S. T. (1999) The epidemic of allergy and asthma. *Nature* **402**(Suppl), B2–4.
2. Howarth, P. H. (1998) Is allergy increasing?—early life influences. *Clin. Exp. Allergy* **28**(Suppl 6) 2–7.
3. (1998) Forecasted state specific estimates of self-reported asthma prevalence. *MMWR Morb. Mortal. Wkly. Rep.* **47**, 1022–1025.
4. Weinberger, M. (2000) Zafirlukast and cromolyn are effective first-line therapies for child asthma. *Ann. Allergy Asthma Immunol.* **84**, 638–639.
5. Kemp, J. P. (2000) Guidelines update: where do the new therapies fit in the management of asthma? NHLBI and WHO Global Initiative for Asthma. *Drugs* **59**(Suppl 1):23–28; discussion 43–25.
6. Creticos, P. S., Marsh, D. G., Proud, D., et al. (1989) Responses to ragweed-pollen nasal challenge before and after immunotherapy. *J. Allergy Clin. Immunol.* **84**, 197–205.
7. Van Metre, T. E. and Adkinson, N. F. (1993) Immunotherapy for aeroallergen disease, in *Allergy: Principles and Practice*. 4th Edition. (Middleton E., ed.) Mosby, St. Louis, MO, pp. 1109–1136.
8. Nelson, H. S., Lahr, J., Rule, R., Bock, A., and Leung, D. (1997) Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. *J. Allergy Clin. Immunol.* **99**, 744–751.
9. Horner, A. A., Van Uden, J. H., Zubeldia, J. M., Broide, D., and Raz, E. (2001) DNA-based immunotherapeutics for the treatment of allergic disease. *Immunol. Rev.* **179**, 102–118.
10. Horner, A. A., Nguyen, M. D., Ronaghy, A., Cinman, N., Verbeek, S., and Raz, E. (2000) DNA-based vaccination reduces the risk of lethal anaphylactic hypersensitivity in mice. *J. Allergy Clin. Immunol.* **106**, 349–356.

11. Hsu, C. H., Chua, K. Y., Tao, M. H., Huang, S. K., and Hsieh, K. H. (1996) Inhibition of specific IgE response in vivo by allergen-gene transfer. *Int. Immunol.* **8**, 1405–1411.
12. Kline, J. N., Waldschmidt, T. J., Businga, T. R., et al. (1998) Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555–2559.
13. Raz, E., Tighe, H., Sato, Y., et al. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* **93**, 5141–5145.
14. Roman, M., Martin-Orozco, E., Goodman, J. S., et al. (1997) Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
15. Roman, M., Spiegelberg, H. L., Broide, D., and Raz, E. (1997) Gene immunization for allergic disorders. *Springer Semin. Immunopathol.* **19**, 223–232.
16. Roy, K., Mao, H. Q., Huang, S. K., and Leong, K. W. (1999) Oral gene delivery with chitosan—DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* **5**, 387–391.
17. Creticos, P. S., Balcer, S. L., Schroeder, J. T., et al. (2001) Initial immunotherapy trial to explore the safety, tolerability, and immunogenicity of subcutaneous injection of the AMB a 1 immunostimulatory oligonucleotide conjugate (AIC) in ragweed allergic adults. *J. Allergy Clin. Immunol.* **107**, S216(A-712).
18. Creticos, P. S., Eiden, J. J., Balcer, S. L., et al. (2000) Immunostimulatory oligodeoxynucleotides conjugated to Amb a 1: safety, skin test reactivity, and basophil histamine release [abstract]. *J. Allergy Clin. Immunol.* **105**, S70.
19. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. *Eur. J. Immunol.* **30**, 1939–1947.
20. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* **106**, 124–134.
21. Kobayashi, H., Horner, A. A., Martin-Orozco, E., and Raz, E. (2000) Pre-priming: a novel approach to DNA-based vaccination and immunomodulation. *Springer Semin. Immunopathol.* **22**, 85–96.
22. Kobayashi, H., Horner, A. A., Takabayashi, K., et al. (1999) Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity. *Cell. Immunol.* **198**, 69–75.
23. Broide, D., Schwarze, J., Tighe, H., et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054–7062.
24. Broide, D. H., Stachnick, G., Castaneda, D., et al. (2001) Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J. Clin. Immunol.* **21**, 175–182.
25. Magone, M. T., Chan, C. C., Beck, L., Whitcup, S. M., and Raz, E. (2000) Systemic or mucosal administration of immunostimulatory DNA inhibits

- early and late phases of murine allergic conjunctivitis. *Eur. J. Immunol.* **30**, 1841–1850.
26. Sur, S., Wild, J. S., Choudhury, B. K., Sur, N., Alam, R., and Klinman, D. M. (1999) Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* **162**, 6284–6293.
 27. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* **29**, 2344–2353.
 28. Fujieda, S., Iho, S., Kimura, Y., Yamamoto, H., Igawa, H., and Saito, H. (2000) Synthetic oligodeoxynucleotides inhibit IgE induction in human lymphocytes. *Am. J. Respir. Crit. Care Med.* **162**, 232–239.
 29. Horner, A. A., Widhopf, G. F., Burger, J., et al. (2001) Immunostimulatory DNA mediated inhibition of IL-4 dependent IgE synthesis from human B cells. *J. Allergy Clin. Immunol.* in press.
 30. Yamamoto, T., Yamamoto, S., Kataoka, T., Komuro, K., Kohase, M., and Tokunaga, T. (1994) Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. *Jpn. J. Cancer Res.* **85**, 775–779.

Immunostimulatory DNA for Allergic Asthma

Reid K Ikeda, Kenji Takabayashi,
and David Broide

1. INTRODUCTION

1.1. The Hygiene Hypothesis and the Development of Asthma

Asthma is a common disease afflicting 17 million Americans, or approx 7% of the United States population (1). Asthma has increased in prevalence and has been associated with increased morbidity and mortality over the past two decades (2). In some developed Western countries, such as Great Britain and Australia, the estimated incidence of asthma is one in four children under the age of 14 (3). Although the reason for the increase in asthma prevalence is not well understood, one of the proposed explanations for the increased incidence of atopy in developed countries is the "hygiene hypothesis." The hygiene hypothesis is based on the principle that, during a critical period of immune system maturation in infants genetically predisposed to atopy and Th2 responses to antigen, a reduction in previously encountered infectious stimuli (particularly with organisms such as mycobacteria, measles, and hepatitis A, which induce strong cell-mediated Th1 immune responses), has altered the Th1/Th2 lymphocyte balance in favor of Th2 responses (4). A reduced incidence of mycobacterial and viral infections is hypothesized to result in decreased IL-12 production by dendritic cells, and a consequent decrease in interferon- γ generating Th1 lymphocytes, which would normally bias the immune response to a Th1 response to newly encountered antigens, and thus, inhibit the release of pro-allergic cytokines, such as IL-4 by Th2 lymphocytes (4). The decrease in childhood infections in industrialized countries is attributed to several developments, most prominently, smaller family size and a concomitant decrease in cross-infection of siblings (5), as well as the increased use of antibiotics, leading to a change in

bacterial colonization of the bowel and altered immunological programming of the gut-associated lymphoid system (6). It is important to note, however, that some epidemiological associations, such as household size and socioeconomic status, do not correlate as strongly with the incidence of asthma, as with other allergic disorders (5). As asthma is precipitated by a number of different stimuli, including inhaled allergens and respiratory viruses, further investigation is required to determine if the hygiene hypothesis is applicable to all variants of asthma.

1.2. Immunostimulatory DNA and the Hygiene Hypothesis

Although the hygiene hypothesis remains unproven thus far, it provides theoretical support for the application of bacterial DNA-derived immunostimulatory sequence-based immunotherapies in asthma. Immunostimulatory DNA sequences (ISS) are short 6 base pair noncoding DNA sequences, containing an unmethylated CpG motif that are enriched in bacterial, compared to vertebrate genomes. These ISS sequences have been found to bias the immune system, to generate Th1 and not Th2 responses, thereby mimicking natural infectious exposures, which according to the hygiene hypothesis, have been reduced in individuals predisposed to atopy. If the hygiene hypothesis is correct, and decreased infectious stimuli are responsible for the observed increase in allergic diseases, ISS-based immunotherapies represent a novel and promising approach to the treatment of asthma.

1.3. Immune Modulation as a Goal of Asthma Therapy

Given the understanding that asthma is an inflammatory disease of the airways, which may lead to remodeling and persistent airway obstruction in some asthmatics if untreated, institution of antiinflammatory therapy in the incipient stages of airway inflammation has been emphasized (7). The most effective current therapy for asthma is corticosteroids, which unfortunately have numerous adverse effects when administered systemically, including osteoporosis, hyperglycemia, adrenal suppression, neuropsychiatric changes, myopathy, cataracts, and growth retardation. Although administration of inhaled corticosteroids are associated with significantly fewer side effects compared to systemic therapy with corticosteroids, concerns have been expressed about prolonged courses of high doses of inhaled corticosteroids inducing osteoporosis, cataracts, and growth suppression. Therefore, efforts have increased to develop immunomodulatory therapies other than corticosteroids to reduce airway inflammation in asthmatics.

Traditional protein-based immunotherapy to treat allergic rhinitis has been used since 1911 (8). Although immunotherapy to common inhaled

allergens, such as grass pollen, ragweed, and house dust mite has reduced symptoms in patients with allergic rhinitis (9), in asthmatics, immunotherapy has been less effective (10–12). Furthermore, patients with moderate to severe asthma, who have the greatest need for improved therapy, have the highest risk of anaphylaxis with traditional protein immunotherapy, limiting the potential use of such treatment in allergic asthma.

Given the limitations of current immunomodulatory therapy, DNA-based therapy for asthma has been recently investigated, and several different approaches have been identified, including DNA gene therapy using allergen exon-coding immunization, noncoding ISS therapy using DNA sequences rich in unmethylated CpG motifs, and most recently, ISS protein allergen conjugate therapy. The differing mechanisms of action and potential efficacy of each DNA-based approach in the treatment of asthma has been investigated extensively in animal models, with some data now available in humans.

2. RESULTS AND DISCUSSION

2.1. DNA-Based Therapy For Asthma

The use of plasmid DNA coding for a specific antigen or allergen protein as a mode of gene therapy has been investigated most extensively in infectious diseases, but also in allergy. In a study demonstrating the potential of such an approach, Wolff et al. injected a DNA construct containing the gene for β -galactosidase (β -gal) into mouse skeletal muscle, and demonstrated the expression of β -gal protein for approx 2 mo (13). Similar studies have shown that plasmid DNA containing the β -gal gene when injected into human skin grafted onto nude mice, results in expression of β -gal protein in human skin (14).

The use of DNA vaccines encoding an allergen to modify the Th2 immune response to that allergen has been studied extensively in mice (15,16). Both humoral and cellular immune responses to encoded allergens can be generated following intramuscular or intradermal injection of plasmid DNA encoding a specific antigen (15,16). Studies by Raz et al. (15) demonstrated that mice immunized intradermally with a plasmid DNA construct containing *lacZ*, the gene encoding β -gal, generated a Th1 immune response, whereas mice immunized with the β -gal protein itself developed a Th2 response. Therefore, the immune response differed depending on whether the same antigen was delivered as a DNA plasmid or as a protein. Because asthma is characterized by a Th2 response to antigen, these findings suggested, the potential use of plasmid DNA encoding allergens to

downregulate the Th2 immune response to allergens by generating a Th1 response. DNA gene vaccination with plasmid DNA encoding β -gal inhibited both IgE and IL-5 cytokine response to β -gal protein in alum (17). Additional studies using a mouse model of asthma demonstrated that mice sensitized to develop a Th2 response to ova protein, and pretreated with three intradermal injections of plasmid DNA encoding ova protein, developed significantly less bronchoalveolar lavage fluid eosinophils (84% inhibition), lung eosinophils (70% inhibition), and bone marrow eosinophils (70% inhibition), after inhalation of ova protein, as compared to mice injected with a control plasmid DNA construct (17). Furthermore, studies using a rat model of asthma have demonstrated that the IgE response, histamine release into bronchoalveolar lavage fluid, and bronchial hyperreactivity, following challenge with aerosolized dust mite allergen Der p5 were inhibited in rats immunized with a plasmid DNA encoding the Der p 5 allergen (18). The response to the DNA vaccine was antigen-specific, as the rats immunized with plasmid DNA encoding the Der p 5 allergen were protected against Der p 5 allergen challenge, but not against a different allergen i.e., ovalbumin challenge.

The mechanism by which plasmid DNA gene immunization induces a Th1 response is probably multifactorial, with both the intracellular processing of the plasmid DNA gene, as well as ISS in the plasmid backbone (19) playing key roles. Studies by Sato et al. (19) demonstrated the importance of noncoding immunostimulatory sequences containing an ISS motif in the plasmid DNA backbone, to the development of Th1 immune responses. DNA vaccines constructed with a plasmid backbone including an *amp* R gene, which contains an ISS motif, developed Th1 responses. In contrast, DNA vaccines constructed with a plasmid backbone containing a *kan* R gene, which does not contain ISS motifs, did not develop Th1 responses (19). The development of a Th1 response was independent of the coding sequences of the DNA vaccine and was thus, determined by the presence or absence of immunostimulatory motifs, which has focused further studies on the potential effect of immunostimulatory DNA sequences, alone as a therapy for asthma.

2.2. Immunostimulatory Sequence DNA Therapy for Asthma

An alternative method of DNA-based immunization, which has received significant attention recently is the use of CpG-rich ISS as inducers of Th1 responses to antigen. Interest in this approach began when Tokunaga et al. (20) demonstrated that the CpG motifs in *Mycobacterium bovis* BCG DNA induced interferon- γ , a cytokine produced by Th1 cells. In contrast, the immunostimulatory effect of vertebrate DNA is significantly lower than that

of bacterial DNA. The decreased immunostimulatory effect of vertebrate DNA is likely related to a combination of the lower frequency of CpG motifs in vertebrate compared to bacterial DNA, as well as a high frequency of cytosine methylation in vertebrate compared to bacterial DNA, which abolishes the immunostimulatory effect of vertebrate CpG sequences. The DNA hexamer sequences generating the optimal Th1 adjuvant effect include the motif 5'-purine-purine-CpG-pyrimidine-pyrimidine-3', e.g., AACGTT or GACGTC (21), which activate the secretion of IFN- γ . ISS has an indirect effect on the Th1 immune response, by activating the innate immune system to upregulate cytokine expression (IL-12, IL-18, IFN- γ , IFN- α , IFN- β), upregulate MHC molecules, and upregulate costimulatory molecules (22–24). Furthermore, ISS induces natural killer (NK) cells to release interferon- γ , and these effects on the innate immune system lead to a cytokine milieu (IFN- γ +, IL-12+), which biases the T lymphocyte immune response to a Th1 response to newly encountered antigens.

Several studies have demonstrated that ISS can inhibit Th2 cytokine responses, as well as eosinophilic inflammation and airway hyperreactivity to methacholine in mouse models of asthma (25–27). Our laboratory has demonstrated that in addition to inhibiting eosinophilia of the airway (by 93%) and lung parenchyma (by 91%), ISS also significantly inhibited blood eosinophilia (86%), suggesting that ISS exerts a significant effect on the bone marrow production and/or release of eosinophils (25). The inhibition of the bone marrow production of eosinophils by 58% was associated with a significant inhibition of T-cell derived cytokine production (IL-5, GM-CSF and IL-3). As IL-5 is an important lineage-specific eosinophil growth factor, as well as an inducer of the bone marrow release of eosinophils, the inhibitory effect of ISS on the generation of IL-5 plays an important role in mediating the effect of ISS on bone marrow production and/or release of eosinophils. The onset of the ISS effect on reducing the number of tissue eosinophils is both immediate (within one day of administration) and is not due to ISS directly inducing eosinophil apoptosis (25). ISS is effective in inhibiting eosinophilic airway inflammation when administered either systemically (intraperitoneally), or mucosally (i.e. intranasally or intratracheally) (25). Interestingly, a single dose of ISS has been shown to reduce airway eosinophilia as effectively as daily injections of corticosteroids for 7 d (25). Furthermore, while both ISS and corticosteroids inhibited IL-5 production, only ISS was able to induce allergen-specific IFN- γ production and redirect the immune system toward a Th1 response.

The administration of ISS through activation of innate immunity, could therefore inhibit pulmonary eosinophilia via several potential mechanisms.

One mechanism by which ISS can reduce pulmonary eosinophilia is by inhibiting T-cell derived cytokines (i.e., IL-5) important to the bone marrow production of eosinophils. ISS exerts this effect indirectly by stimulating NK cells and macrophages to generate IL-12 and IFNs, which subsequently inhibit T-cell generation of IL-5, GM-CSF, and IL-3. The greater reduction by ISS of peripheral blood eosinophilia (86% decrease) compared with reduction of bone marrow eosinophilia (58% decrease) suggests that ISS may have also inhibited release of eosinophils from the bone marrow, perhaps through inhibition of IL-5, which is known to induce release of eosinophils from the bone marrow. Another mechanism by which ISS could reduce eosinophil recruitment is through the generation of cytokines, such as IL-12 (28,29), IFN- γ (30), and IFN- α (31) that have previously been demonstrated to inhibit eosinophilic inflammation in models of allergic inflammation and parasitic infection.

2.3. How Important is ISS Induction of Th1 Responses to the Protective Effect of ISS in Asthma?

The cellular mechanism of action of ISS in vivo may be more complex than that initially hypothesized based on results from in vitro studies. Although ISS induces a strong Th1 response, the antiallergic effect of ISS may not be mediated by this mechanism in vivo. In support of this hypothesis are studies demonstrating that adoptive transfer of antigen specific Th1 cells do not protect against eosinophilic airway inflammation in a mouse model of asthma (32,33), and studies demonstrating that ISS can mediate its antiallergic effect in vivo independent of the Th1 cytokines interferon- γ and IL-12 (34). We have also demonstrated that ISS can inhibit eosinophilic inflammation and airway hyperreactivity in mice depleted of NK cells, a major source of the Th1 cytokine interferon- γ induced by ISS (35). Thus, the ability of ISS to induce a Th1 response and interferon- γ may not be essential to its inhibitory effect on allergic inflammation. However, some studies suggest that the induction of interferon- γ and a Th1 response is essential to the anti-allergic effect of ISS (27).

2.4. Systemic ISS Mediates Reversible Inhibition of the Th2 Response

Recently, we have investigated whether ISS induces a temporary or sustained inhibition of Th2 responses to inhaled antigen (36). Ova sensitized mice received a single dose of ISS, 1 d prior to ova inhalation challenge and were subsequently rechallenged with ova inhalation at varying time intervals (1–8 wk later) to determine whether the initial ISS dose had a protective

effect against the development of a Th2 response several weeks later. The single ISS dose was shown to inhibit BAL eosinophilia by 89%, and lung eosinophilia by 77% at 1 wk. This inhibitory effect waned with time, however, as BAL eosinophilia was decreased by 77% at 2 wk, 48% at 4 wk, 29% at 6 wk, and not reduced at all by 8 wk after ISS administration. Lung eosinophilia was significantly inhibited at 2 and 4 wk by ISS, but no statistically significant reduction was demonstrated at 8 wk. Likewise, ISS inhibited bone marrow eosinophilia (by 46%) and peripheral blood eosinophilia (by 63%) at 1 wk, but not at 8 wk. ISS inhibited airway hyperreactivity to methacholine at 1 wk, 2 wk, and 4 wk, but by 6 wk, statistically significant inhibition was not demonstrated. ISS also significantly inhibited IgE levels 2 wk after ISS administration, but IgE was not reduced at 4 wk or at 6 wk. Finally, ISS significantly inhibited IL-5 at 1 wk by 78%, 51% at 4 wk, and 38% at 8 wk, while significantly inducing IFN- γ production at 1 wk, but not at 8 wk. These results indicate that while a single dose of ISS is very effective in inhibiting IL-5, eosinophilic airway inflammation, and bronchial hyperresponsiveness, the duration of inhibition of Th2 responses is not permanently sustained. The reversible inhibition of Th2 responses at 8 wk suggests that it may be necessary to administer ISS repeatedly at monthly intervals to maintain inhibition of airway inflammation and airway hyperreactivity.

2.5. ISS: Protein Allergen Conjugates

Recently, interest has developed in the use of allergen protein conjugated to ISS for the treatment of allergic diseases, as this approach has certain theoretical advantages compared to unconjugated ISS. The primary advantage of allergen protein conjugated ISS, is that physically linking ISS to antigens would increase the likelihood of their delivery to the same antigen presenting cell (APC) resulting in an amplified, antigen-specific Th1 immune response (37), which is much less likely to occur when ISS and antigen are administered separately. The same APC would therefore process the antigen and present the antigen to Th cells. While ISS would induce the APC to release IL-12, thus biasing the well-targeted Th cell to differentiate toward a Th1 phenotype. Furthermore, theoretically, the ISS allergen protein conjugate might be less allergenic than the ISS allergen protein mixture, as steric hindrance or electrostatic blockade of IgE binding epitopes could occur with ISS coating the allergen (37).

Given the poor immunogenicity and risk of anaphylaxis with current allergen protein immunotherapy, Tighe et al. analyzed the immune response to Amb 1, the major short ragweed allergen, conjugated to ISS in mice,

rabbits, and monkeys (37). In mice, injection of AmB a 1 alone, generated a Th2 response characterized by high IL-5 production and low levels of IFN- γ . Whereas injection of the AmB a 1-ISS conjugate induced a Th1 response, characterized by high levels of IFN- γ production and low levels of IL-5. Injection of a mixture of AmB a 1 and ISS was much less effective than the AmBa 1-ISS conjugate in generating a Th1 immune response. Furthermore, in mice primed for a Th2 response with injections of AmB a 1 in alum, the AmB a 1-ISS conjugate induced a Th1 immune response and reversed the preexisting Th2 biased immune deviation. The AmB a 1-ISS conjugate also prevented an increase in IgE antibody formation after challenge with AmB a 1, and inhibited the release of histamine from human basophils in patients with ragweed allergy *in vitro* by 30-fold. Whereas a mixture of AmB a 1 and ISS did not suppress histamine release. These results demonstrate that allergen conjugated to ISS can enhance the Th1 immune response, reverse a preexisting Th2 immune bias, and potentially reduce the allergenicity of protein immunotherapy.

In a mouse model of asthma concomitant administration of antigen and CpG intratracheally, prior to allergen challenge reduced airway eosinophilia and airway hyperreactivity to methacholine, downregulated the Th2 immune response, and enhanced Th1 cytokine expression (38). The ova-conjugated CpG was found to be 100-fold more efficient than the unconjugated mixture in reducing airway eosinophilia and bronchial hyperreactivity, with this inhibitory effect observed for approx two mo. ISS allergen protein conjugates therefore offer significant promise as therapy for asthma and other allergic disorders, although investigation of its immune modulating properties in humans requires further study.

3. CONCLUSION

A variety of promising DNA-based immunomodulatory therapies for asthma are currently being investigated. Exon-coding DNA vaccines are designed to inhibit the Th2 immune response to specific DNA encoded allergens. This mode of DNA-based therapy requires knowledge of the DNA coding sequence of the allergen administered and tends not to induce as strong an immune response as protein antigens. Therapy with ISS attempts to redirect the host immune response from a Th2 to a Th1 response. ISS allergen protein conjugate therapy is designed to improve the efficacy of unconjugated ISS therapy by targeting ISS, and the protein allergen to the same antigen presenting cell. Ongoing clinical trials in human subjects will determine if these DNA-based therapies are safe and effective in human asthma.

ACKNOWLEDGMENTS

The authors acknowledge the important contribution of Eyal Raz MD to the studies of ISS and asthma performed at UCSD. This research was supported by NIH grant AI40682, and an American Lung Association Research Fellowship Award to Reid Ikeda, MD.

REFERENCES

1. CDC (1998) Forecasted state-specific estimates of self-reported asthma prevalence-United States, 1998. *MMWR* **47**, 1022.
2. CDC (1996) Asthma mortality and hospitalization among children and young adults-United States, 1980-1993. *MMWR* **45**, 350.
3. Beasley, R. (1998) Worldwide variations in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis and atopic eczema. *Lancet* **351**, 1225.
4. Holgate, S. T. (1999) The epidemic of allergy and asthma. *Nature*. **402** (6760 Suppl):B2.
5. Strachan, D. P. (2000) Family size, infection and atopy: the first decade of the "hygiene hypothesis." *Thorax* **55**(Suppl 1):S2.
6. Hopkin, J. M. (1997) Mechanisms of enhanced prevalence of asthma and atopy in developed countries. *Curr. Opin. Immunol.* **9**, 788.
7. NIH (1997) Guidelines for the diagnosis and management of asthma. NIH Publication 97-4051:3
8. Noon, L. (1911) Prophylactic inoculation against hay fever. *Lancet* **I**:1572.
9. Adkinson, N. F., Eggleston, P. A., et al. (1997) A controlled trial of immunotherapy for asthma in allergic children. *N. Engl. J. Med.* **336**, 324.
10. Creticos, P. S. (1997) Immunotherapy. In: Allergy. 2nd ed. Kaplan AP, ed. Saunders; Philadelphia pp. 726-739.
11. Norman, P. S. (1998) Immunotherapy past and present. *J. Allergy Clin. Immunol.* **102**, 1.
12. Van Metre, T. E., and Adkinson, N. F. (1993) Immunotherapy for aeroallergen disease. In: Middleton E, editor. Allergy: Principles and practice. 4th ed. Mosby, St. Louis, pp.1109-1136.
13. Wolff, J. A., Malone, R. W., Williams, P., et al. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465.
14. Hengge, U. R., Walker, P. S., and Vogel, J. C. (1996) Expression of naked DNA in human pig, and mouse skin. *J. Clin. Invest.* **97**, 2911.
15. Raz, E., Tighe, H., Sato, Y., et al. (1996) Preferential induction of a TH1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* **93**, 5141.
16. Weiner, D. B., and Kennedy, R. C. (1999) Genetic vaccines. *Sci. Am* **281**, 34.
17. Broide, D., Orozco, E. M., Roman, M., Carson, D. A., and Raz, E. (1997) Intradermal gene vaccination down-regulates both arms of the allergic response. *J. Allergy Clin. Immunol.* **99**, S129.
18. Hsu, C. H., Chua, K. Y., Tao, M. H., et al. (1996) Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat. Med.* **2**, 540.

19. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352.
20. Yamamoto, S., Kuramoto, E., Shimada, S., Tokunaga, T. (1988) In vitro augmentation of natural killer cell activity and production of interferon- α/β and γ with deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. *Jpn. J. Cancer Res.* **79**, 866.
21. Yamamoto, T., Yamamoto, S., Kataoka, T., Komuro, K., Kohase, M., and Tokunaga, T. (1994) Synthetic oligonucleotides with certain palindromes stimulate interferon production of human peripheral blood lymphocytes in vitro. *Jpn. J. Cancer Res.* **85**, 775.
22. Krieg, A. M., Yi, A. K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546.
23. Roman, M., Orozco, E. M., Goodman, J., et al. (1997) Immunostimulatory DNA sequences function as T helper-1 promoting adjuvants. *Nat. Med.* **3**, 849.
24. Yamamoto, S., Yamamoto, T., Kataoka, T., Kuramoto, E., Yano, O., and Tokunaga, T. (1992) Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and IFN-mediated natural killer activity. *J. Immunol.* **148**, 4072.
25. Broide, D., Schwarze, J., Tighe, H., et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054.
26. Kline, J. N., Waldschmidt, T. J., Businga, T. R., et al. (1998) Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555.
27. Sur, S., Wild, J. S., Choudhury, B. K., Sur, N., Alam, R., and Klinman, D. M. (1999) Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* **162**, 6284.
28. Gavett, S. H., O'Hearn, D. J., Li, X., Huang, S. K., Finkelman, F. D., and Wills-Karp, M. (1995) Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* **182**, 1527.
29. Trinchieri, G. (1995) Interleukin-12: a proinflammatory cytokine with immuno-regulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* **13**, 251.
30. Nakajima, H., Nakao, A., Watanabe, Y., Yoshida, S., and Iwamoto, I. (1994) IFN- α inhibits antigen-induced eosinophil and CD4⁺ T cell recruitment into tissue. *J. Immunol.* **153**, 1264.
31. Iwamoto, I., Nakajima, H., Endo, H., Yoshida, S. (1993) Interferon γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J. Exp. Med.* **177**, 573.
32. Hansen, G., Berry, G., DeKruyff, R. H., and Umetsu, D. T. (1999) Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperactivity but cause severe airway inflammation. *J. Clin. Invest.* **103**, 175.

33. Randolph, D. A., Stephens, R., Carruthers, C. J., and Chaplin, D. D. (1999) Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J. Clin. Invest.* **104**, 1021.
34. Kline, J. N., Krieg, A. M., Waldschmidt, T. J., Ballas, Z. K., Jain, V., and Businga, T. R. (1999) CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J. Allergy Clin. Immunol.* **104**, 1258.
35. Broide, D. H., Stachnick, G., Castaneda, D., et al. (2001) Immunostimulatory DNA mediates inhibition of eosinophilic inflammation and airway hyperreactivity independent of NK cells in vivo. *J. Allergy Clin. Immunol.*, **108**, 759–763.
36. Broide, D. H., Stachnick, G., Castaneda, D., et al. (2001) Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J. Clin. Immunol.* **21**, 163.
37. Tighe, H., Takabayashi, K., Schwartz, D., et al. (2000) Conjugation of immunostimulatory DNA to the short ragweed allergen Amb a 1 enhances its immunogenicity and reduces its allergenicity. *J. Allergy Clin. Immunol.* **106**, 124–134.
38. Shirota, H., Sano, K., Kikuchi, T., Tamura, G., and Shirato, K. (2000) Regulation of murine airway eosinophilia and Th2 cells by antigen-conjugated CpG oligodeoxynucleotides as a novel antigen-specific immunomodulator. *J. Immunol.* **164**, 5575.

CpG Oligodeoxynucleotides in Asthma

Kunihiko Kitagaki and Joel N. Kline

1. CURRENT UNDERSTANDING OF ASTHMA: IMPLICATIONS OF BALANCE BETWEEN TH1 AND TH2

Asthma is recognized as chronic airway inflammation characterized by airway eosinophilic inflammation and airway hyperreactivity (1). It is also highly correlated with atopy, and antigen-induced immunoglobulin E (IgE) production is increased in patients with allergic asthma (2). Interleukin-5 (IL-5) is the terminal differentiation, activation, and survival factor for eosinophils (3,4). IL-4 amplifies the asthmatic inflammatory response through IgE class switching (5) and stimulating growth of mast cells (6). IL-13 also plays a role in IgE synthesis (7). CD4-positive T lymphocytes can be divided in to type 1 helper T (Th1) and type 2 helper T (Th2) cells on the basis of their cytokine production pattern (8). Th2 cells can produce IL-4, IL-5, and IL-13. Therefore, although mast cells and eosinophils also can secrete these cytokines (9,10), Th2 cells are seen as having a central role for pathogenesis of asthma since they produce these proinflammatory mediators in antigen-specific responses (11). Th1 cells produce IL-2 and interferon γ (IFN- γ) and none of the Th2 cytokines. Th1 and Th2 cells are counter-regulatory through their cytokine production. For example, IL-4 promotes Th2 development (12) and inhibits Th1 cells (13). In contrast, IFN- γ promotes Th1 development (12) and inhibits the proliferation of Th2 cells (14) and the synthesis of IL-4 and IL-5 by Th2 cells (15). Th1 and Th2 cells have been identified in humans (16–18). These lines of evidences suggest that inhibition of Th2 cytokine production or enhancement of Th1 cytokine production may be useful in the treatment of asthma.

2. CURRENT THERAPY FOR ASTHMA

Because asthma is characterized by chronic airway inflammation, local or systemic antiinflammatory medications are mandated for all patients with persistent asthma (1). Although some mediator antagonists such as the 5'-lipoxygenase inhibitors and the leukotriene receptor antagonists are useful antiinflammatory drugs (19), inhaled glucocorticosteroids remain the gold standard for treatment of airway inflammation in asthma. Inhibition of inflammatory gene expression is considered as the mechanism of antiinflammatory effects of glucocorticosteroids (20). However, these drugs also inhibit production of the Th1 cytokine, IFN- γ (21); this could theoretically lead to enhanced Th2 activity, which is likely to negatively influence asthma control.

At least 15 million Americans suffer from asthma, which is a common increasingly prevalent disease in industrialized countries. A recent total annual cost for asthma is estimated at approx \$6 billion in the United States (22). Despite substantial international efforts to the contrary, prevalence, morbidity and mortality from asthma have increased during the past three decades (23). Moreover, recent studies suggest that airway remodeling is an important factor late in the course of asthma. These factors suggest that current therapy is inadequate to control asthma.

3. IMMUNOSTIMULATORY DNA; CpG-ODN AS A Th1 TRIGGER

In 1995, Krieg et al. reported that DNA motifs containing a central unmethylated CpG dinucleotide flanked by two 5'-purines and two 3'-pyrimidines cause B-cell activation (24). In bacterial DNA, these motifs are observed with high frequency. On the other hand, in mammalian DNA, these motifs are present at about only one quarter of the frequency expected if base utilization was random, and the cytosines are usually methylated (25). It has been known that vaccination with naked DNA elicits cellular and humoral immune responses against the encoded proteins (26,27). In 1996, Sato et al. found that CpG motifs are necessary for effective DNA vaccine, and oligonucleotides (ODN) containing these motifs produce proinflammatory cytokines like IFN- α , IFN- β , and IL-12 (28).

The signaling pathways through which CpG-ODN have their effects are still unclear, and are the topic of considerable current study. Because several ODN exert biological effects on B cells even after covalent coupling to sepharose beads, it might be expected that effects of CpG-ODN require interactions with a cell surface receptor (29). Recently, it was reported that Toll-like receptor 9 (TLR9) deficient mice did not show any response to CpG-ODN (30), suggesting that TLR9 must be an important ligand for CpG-

ODN. On the other hand, it has been reported that lysosomotropic agents like bafilomycin A and chloroquine completely inhibited CpG-ODN-induced biological responses (31–33). Therefore, it is also thought that translocation of CpG-ODN into endosomes is necessary for the activation of antigen presenting cells (APCs) (34). Although there is debate over the initial step of the response to CpG-ODN, the transcription factors AP-1 and NF κ B signal pathways are clearly activated by CpG-ODN in antigen-presenting cells (31–33,35). CpG-ODN cannot activate NF- κ B in mice lacking MyD88 which is the adaptor protein for TLRs (36).

CpG-ODN induce rapid production of IFN- γ from natural killer (NK) cells, a process which is highly dependent on macrophage-derived IL-12 (37). Dendritic cells, which are a key player for the induction of antigen-presentation to naive T cells also produce a large amount of IL-12 following activation by CpG-ODN (38). Recently, it reported that CpG-ODN induce secretion of IL-18 from peripheral blood mononuclear cells of allergic patients (39). Thus, CpG-ODN have profound effects on the induction of Th1 responses.

4. EPIDEMIOLOGY OF ASTHMA

Recent epidemiological studies have discovered that the prevalence and morbidity of asthma and other allergic diseases are increasing worldwide, but that asthma is more common in Western countries than in developing countries (40). Additionally, the prevalence of asthma is increasing in developing countries as they become more Westernized or urbanized. The reasons for these changes have been obscure.

One hypothesis blamed increasing air pollution as a major risk factor for developing asthma. However, von Mutius et al. reported a lower rate of asthma and allergic disorders in children of Munich than in Leipzig, although Leipzig in former East Germany was more polluted than Munich in former West Germany (41). In a follow-up study, they found the high rate of atopy in children without siblings (42). Since the older siblings bring home infections from school or daycare, this might suggest that the early-life infections prevent the later development of atopy. Their earlier study might support this hypothesis because the Leipzig children were more likely to be in out-of-home daycare than the Munich children. A more direct evidence of the relationship between infection and atopy was shown by Shaheen et al. (43). After an epidemic of measles in Guinea-Bissau in 1979, the risk of atopy was significantly reduced. Moreover, positive tuberculin responses have been found to protect against asthma and atopy in Japanese schoolchildren (44). These data have been interpreted as suggesting that, a bias toward a

Th2 response against environmental allergens may become dominant without exposure to viral or prokaryotic DNA, during childhood infections. Diminished childhood infections in industrialized societies may be responsible for a greater tendency toward Th2 immune deviation, and the increased prevalence of asthma and atopy. Exposure to CpG DNA may be a means of redressing this imbalance.

5. CpG-ODN IN ASTHMA

Based on the above epidemiological findings, and our understanding of CpG-ODN as a Th1 trigger, we examined the effects of CpG-ODN on an allergen-induced model of asthma in mice (45). As we expected, CpG-ODN, but not a control ODN, inhibited allergen-induced airway eosinophilia (Fig. 1A, Fig. 2), airway hyperreactivity (Fig. 1B) and IgE production in association with elevation of Th1 cytokines (IFN- γ and IL-12) and reduction of Th2 cytokine (IL-4) in bronchoalveolar lavage fluid (BALF) (Table 1). Subsequently, the authors and other groups have confirmed the effects of CpG-ODN in other murine models of asthma using a variety of antigens and strains of mice (46–49). These data suggested that the effects of CpG-ODN in murine asthma are neither strain, nor model dependent.

Importantly, CpG-ODN are effective when administered both during and after sensitization. These data might suggest that CpG-ODN have capacities not only to shift an uncommitted Th0 to a Th1 response at the initial immune response but also to overcome a preexisting committed Th2 response. Recently, the latter effect was confirmed by administration of CpG-ODN to asthmatic mice, which have established airway eosinophilia (50). Even if CpG-ODN are administered after sensitization, the inhibitory effects on allergic airway inflammation are long-lasting (48). Although the inhibitory effects of CpG-ODN on inflammatory and other responses in murine models of asthma have been demonstrated consistently, a variety of routes of administration have been examined. CpG-ODN are effective when it is administered systemically (intraperitoneally, intravenously, or subcutaneously), mucosally (i.e., intranasally or intratracheally) and even orally. It is not clear yet whether systemically effective levels of CpG-ODN are necessary to exert inhibitory effects, or whether local responses are sufficient. Broide et al. reported that even when CpG-ODN (100 μ g/animal) were administered either intranasally or intratracheally, bone marrow production of eosinophils was suppressed in parallel with inhibition of airway eosinophilia, demonstrating that systemic responses can be overcome through this route (47). Moreover, Serbrisky et al. found that when CpG-ODN (30 μ g/animal) were administered intraperitoneally, the inhibitory effects on air-

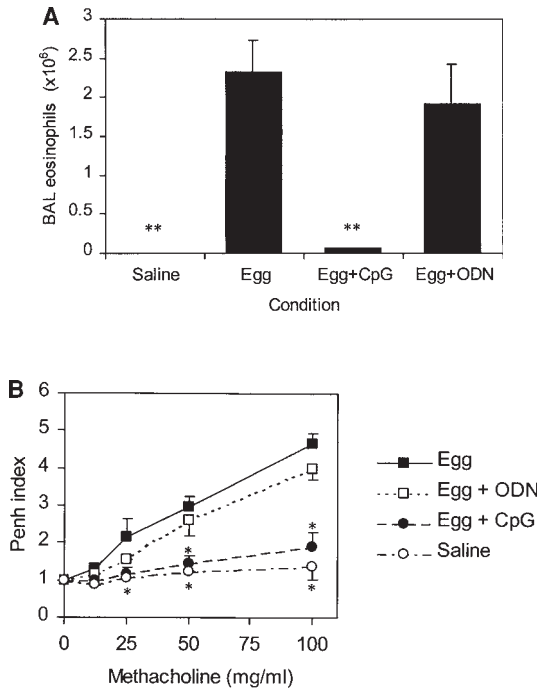


Fig. 1. CpG-ODN prevent the development of airway eosinophilia (**A**) and bronchial hyperreactivity, to inhaled methacholine (**B**) in a murine model of asthma. C57BL/6 mice were exposed to schistosome eggs with or without ODN (30 mg ip, day 0), or saline alone. Mice were challenged in the airway with soluble egg antigen (SEA) (10 μ g) on d 7 and 14.

A. Mice underwent airway lavage at 6 hours following the second challenge. No eosinophils were identified in the BALF of control mice at any time point; marked airway eosinophilia was induced by schistosome egg sensitization and challenge and was prevented by coadministration of CpG-ODN, but not control ODN. **B.** Twenty-four hours after the second exposure to SEA, the mice were placed in whole body plethysmographs and underwent methacholine challenge (between 12.5 and 100 mg/mL). The Penh index is a calculated measure of bronchospasm ($\text{Penh} = [(\text{Te}/0.3\text{Tr}) - 1] \times [2\text{Pef}/3\text{Pif}]$, where Penh = enhanced pause, Te = expiratory time (s), Tr = relaxation time (s), Pef = peak expiratory flow (mL), and Pif = peak inspiratory flow (mL/s). The mice sensitized to schistosome eggs without ODN, or in the presence of control ODN, exhibited significant bronchial reactivity to inhaled methacholine, compared with control mice, or mice exposed to schistosome eggs in the presence of CpG-ODN. Each data point represents the mean $\pm$ SEM of at least four individual experiments. * $p < 0.05$, ** $p < 0.01$ vs the schistosome-egg-sensitized group.

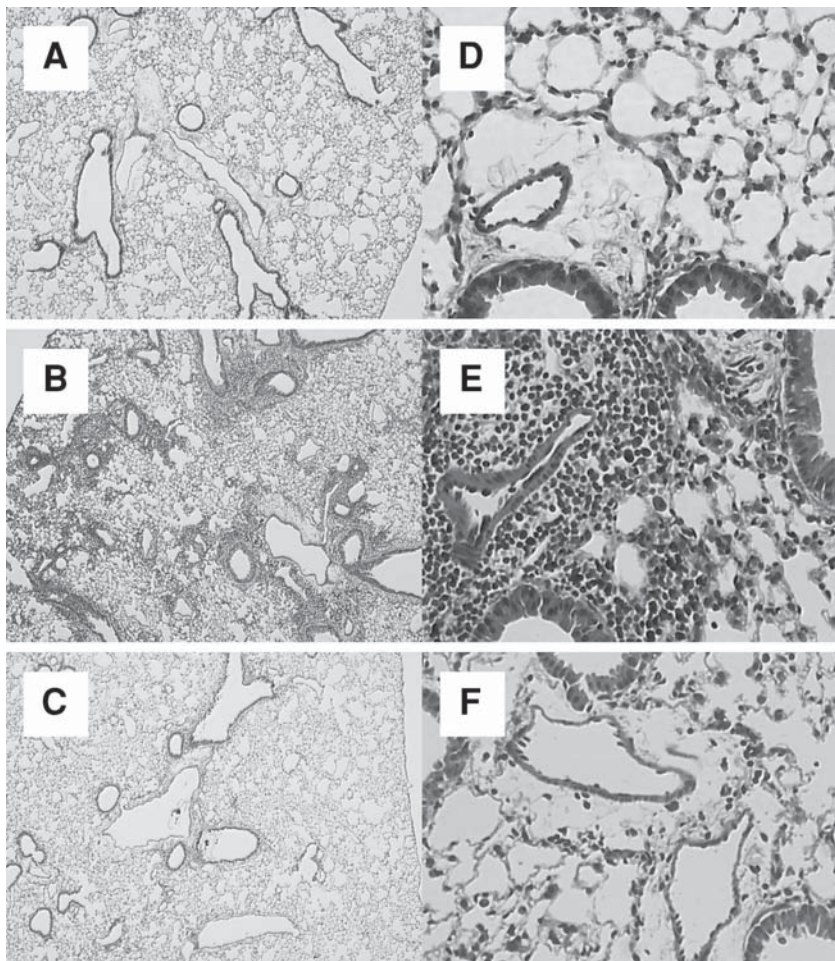


Fig. 2. CpG-ODN prevent the development of peribronchial eosinophilia in a model of asthma. Compared with saline control mice (**A, D**), those mice who are sensitized to schistosome eggs and then are challenged with soluble egg antigen in the airway (**B, E**), develop marked peribronchial-eosinophilic inflammation and epithelial activation, which is markedly reduced in CpG-ODN-treated (**C, F**), but not control-ODN-treated (not shown) mice. **A-C**, $\times 160$; **D-F**, $\times 1000$.

way hyperreactivity were greater, compared with same dose by intratracheal administration (50). However, Shirota et al. reported that lower doses (5 μg /animal) of CpG-ODN administered mucosally reduced allergen-induced airway inflammation with minimal effects on systemic IgE production (49). In

Table 1
Th 1 and Th2 Cytokine Levels in Bronchoalveolar Lavage Fluid

Condition of sensitization	IL-4 (pg/ml)	IFN- γ (pg/ml)	IL-12 (pg/ml)
Saline	5.3 $\pm$ 2.1*	8.4 $\pm$ 3.1	6.9 $\pm$ 3.4
Schistosome egg	115.8 $\pm$ 24.9	15.2 $\pm$ 15.2	18.3 $\pm$ 4.6
CpG-ODN + egg	23.6 $\pm$ 4.7*	403.2 $\pm$ 107.5**	57.6 $\pm$ 19.7**
Control ODN + egg	82.3 $\pm$ 17.6	107.6 $\pm$ 82.4	20.4 $\pm$ 7.1

* $p < 0.05$, ** $p < 0.01$ vs schistosome-egg-sensitized mice.

addition, they also reported that coadministration of antigen is necessary for inhibition of airway inflammation by CpG-ODN. Sur et al. reported that CpG-ODN have to be administered at approx 15 h before antigen challenge, but not at the same time to induce inhibitory effects (48). This has not been found true by other investigators, and the authors have demonstrated that administration of CpG-ODN at the time of sensitization to allergen, as well as any time prior to airway challenge, is effective in preventing asthmatic inflammatory responses (45).

6. INHIBITORY MECHANISMS OF CPG-ODN IN ASTHMA

As described previously, CpG-ODN are strong Th1 inducers, and Th1 cytokines can inhibit Th2 immune responses. In previous studies, it has been reported that treatment of IFN- γ or IL-12, before antigen challenge in sensitized mice, inhibits airway inflammation including, increase of Th2 cytokines and eosinophils, and airway hyperreactivity (51,52). Therefore, enhancing a bias to Th1 type immune responses though IFN- γ and IL-12 production might have explained the inhibitory effects on asthma by CpG-ODN. Indeed, the findings that CpG-ODN failed to inhibit allergen-induced eosinophil recruitment in the airway of IFN-g knock-out (KO) mice might support this hypothesis (48). In contrast to that study, however, we have reported that Th1 cytokines are not required for CpG-ODN to exert protective effects against allergen-induced airway eosinophilia, and airway hyperreactivity because CpG-ODN are effective even in the absence of both IFN- γ and IL-12 (53). We did find that the inhibitory effects of CpG-ODN in IFN- γ /IL-12 double KO mice are reduced in comparison with wild-type mice; approx one log higher amounts of CpG-ODN are required in Th1-deficient mice to develop equivalent levels of protection (measured by reduction in airway eosinophilia), as is seen in wild-type mice. This is one possible explanation of the discrepancy between our results and those of Sur et al. In addition, the oligodeoxynucleotides used by the two groups differ in sequence, and potentially in potency.

Additional data also support that Th1-type responses are not necessary, nor the unique mechanism through which CpG-ODN are effective in blocking Th2-mediated pathology. It has been reported that CpG-ODN and heat-killed *Brucella abortus*, (which contains CpG-motif in its DNA) can diminish Th2 type immune responses independently of their effects on Th1 cytokines (54,55). Recently, data have been accumulating that induction of IFN- γ and IL-12 is not necessary to inhibit allergic disease and Th2 cytokines in immunotherapy (56,57). Even in some murine models of asthma, Th1 cells did not inhibit airway inflammation, but rather appears to worsen it (58–60).

Theoretically, to modulate immune responses from Th2 to Th1 type, the proliferation and activation of Th2 type cells have to be inhibited. Whereas Th1 type cells must differentiate from Th0 cells. Therefore, it might be possible that CpG-ODN have direct abilities to downregulate Th2 type immune responses. CpG-ODN can induce IFN- α and IL-10 (55,61). These two cytokines are known to inhibit antigen-induced eosinophil recruitment in the airways of sensitized mice (62,63). It is unlikely that IFN- α plays a critical role because CpG-ODN are effective in modulating allergen-induced asthma in mice deficient in the type I IFN receptor (53). Induction of IL-10 by CpG-ODN is a reasonable candidate for an inhibitory mediator of asthma by CpG-ODN, since the inhibition of schistosome egg-induced Th2 response by CpG-ODN involves IL-10 (54). In addition, induction of IL-10 is considered to be one of postulated mechanisms for the therapeutic effects of immunotherapy for allergic diseases (64, 65). It is also possible that CpG-ODN induce other cytokines, such as transforming growth factor β , which are thought to play a role in inhibiting airway inflammation in asthma (66).

It is highly likely that CpG-ODN can exert some of its biological effects, especially on T-cell responses, by modulating cell-to-cell interactions. Potential mechanisms through which this may occur may include modulation of costimulatory molecules like CD40, CD80 (B7.1) and CD86 (B7.2) on APCs (50,61,67–69). These outcomes may contribute to the inhibitory effects of CpG-ODN on asthma since T-cell activation is controlled not only by signaling from antigen presented in the context of MHC Class II, but also from costimulatory molecules on APCs. Downregulation of Th1 cytokines independent of Th2 responses are reportedly mediated by alterations in the B7.2-CD28 pathway (55). In another study, B7.1 mRNA was increased and B7.2 mRNA was decreased in the lungs of experimental asthmatic mice by CpG-ODN treatment (50). Although the relationship between CpG-ODN effects and alterations of costimulatory molecules on APCs are still unclear, further studies are necessary to fully understand the role of costimulatory molecules on CpG's effects in asthma and atopy.

7. FUTURE OF CPG-ODN THERAPY FOR ASTHMA

Although large amounts of CpG-ODN themselves can exert anti-asthma effects by IL-12 production, the Th1-promoting effect of IL-12 may be insufficient to suppress a Th2 recall response (70). However, CpG-ODN may act through direct, Th1-independent suppression of Th2 responses. Thus CpG-ODN may be useful for the treatment of all forms of asthma in which Th2 responses are activated, not exclusively on allergen-initiated atopic asthma.

Immunotherapy is currently a little-used alternative asthma treatment. It is the only method of therapy which is thought to act, at least in part, through alteration of Th2-type to Th1-type immune responses. Although the efficacy of immunotherapy for asthma has been difficult to prove, controlled studies support use of immunotherapy for many cases of allergic asthma (71,72). In the past, bacterial vaccines of heat-killed organisms were used as adjuvants with allergen immunotherapy (73). These adjuvants most likely contained CpG motifs, which probably accounts for their immunoactivity. CpG-ODN may be a far more effective adjuvant for immunotherapy. To clarify the mechanisms of immunotherapy for asthma and to make better adjuvants, further studies are necessary.

REFERENCES

1. National Asthma Education and Prevention Program. (1997) Expert panel report2: guidelines for the diagnosis and management of asthma. National Institutes of Health, MD, Publication No. 97-4051.
2. Romagnani, S. (2000) The role of lymphocytes in allergic disease. *J. Allergy Clin. Immunol.* **105**, 399-408.
3. Yamaguchi, Y., T. Suda, S. Ohta, K. Tominaga, Y. Miura, and T. Kasahara. (1991) Analysis of the survival of mature human eosinophils: interleukin-5 prevents apoptosis in mature human eosinophils. *Blood* **78**, 2542-2547.
4. Yamaguchi, Y., T. Suda, J. Suda, M. et al. (1988) Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. *J. Exp. Med.* **167**, 43-56.
5. Del Prete, G., E. Maggi, P. Parronchi, I. et al. (1988) IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *J. Immunol.* **140**, 4193-4198.
6. Saito, H., K. Hatake, A. M., Dvorak, K. M. et al. (1988) Selective differentiation and proliferation of hematopoietic cells induced by recombinant human interleukins. *Proc. Natl. Acad. Sci. USA* **85**, 2288-2292.
7. Punnonen, J., G. Aversa, B. G. Cocks, A. N. et al. (1993) Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc. Natl. Acad. Sci. USA* **90**, 3730-3734.
8. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. (1986) Two types of murine helper T cell clone. I. Definition accord-

- ing to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348–2357.
9. Okayama, Y., C. Petit-Frere, O. Kassel, A. et al. (1995) IgE-dependent expression of mRNA for IL-4 and IL-5 in human lung mast cells. *J. Immunol.* **155**, 1796–1808.
 10. Alam, R., P. Forsythe, S. Stafford, and Y. Fukuda. (1994) Transforming growth factor beta abrogates the effects of hematopoietins on eosinophils and induces their apoptosis. *J. Exp. Med.* **179**, 1041–1045.
 11. Kay, A. B. (1996) TH2-type cytokines in asthma. *Ann N Y Acad. Sci.* **796**, 1–8.
 12. Swain, S. L., Weinberg, A. D. English, M. and Huston, G. (1990) IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* **145**, 3796–3806.
 13. Parronchi, P., M., De Carli, R. Manetti, C. et al. (1992) IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. *J. Immunol.* **149**, 2977–2983.
 14. Gajewski, T. F. and Fitch, F. W. (1988) Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN- gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* **140**, 4245–4252.
 15. Trinchieri, G. (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* **13**, 251–276.
 16. Wierenga, E. A., M. Snoek, C. de Groot, I. et al. (1990) Evidence for compartmentalization of functional subsets of CD2+ T lymphocytes in atopic patients. *J. Immunol.* **144**, 4651–4656.
 17. Del Prete, G. F., De Carli, M., Mastromauro, C. et al. (1991) Purified protein derivative of Mycobacterium tuberculosis and excretory- secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* **88**, 346–350.
 18. Romagnani, S. (1991) Human TH1 and TH2 subsets: doubt no more. *Immunol. Today* **12**, 256–257.
 19. Drazen, J. M., Israel, E. and O'Byrne, P. M. (1999) Treatment of asthma with drugs modifying the leukotriene pathway. *N. Engl. J. Med.* **340**, 197–206.
 20. Barnes, P. J. (1998) Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci. (Colch)* **94**, 557–572.
 21. Arya, S. K., Wong-Staal, F. and Gallo, R. C. (1984) Dexamethasone-mediated inhibition of human T cell growth factor and gamma-interferon messenger RNA. *J. Immunol.* **133**, 273–276.
 22. Smith, D. H., Malone, D. C., Lawson, K. A., Okamoto, L. J., Battista, C. and Saunders, W. B. (1997) A national estimate of the economic costs of asthma. *Am. J. Respir. Crit. Care Med.* **156**, 787–793.
 23. Evans, R., 3rd, Mullally, D. I., Wilson, R. W., et al. (1987) National trends in the morbidity and mortality of asthma in the US. Prevalence, hospitalization and death from asthma over two decades: 1965–1984. *Chest*. **91**, 65S–74S.
 24. Krieg, A. M., Yi, A. K., Matson, S. et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.

25. Bird, A. P. (1986) CpG-rich islands and the function of DNA methylation. *Nature* **321**, 209–213.
26. Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C. and Robinson, H. L. (1993) DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* **90**, 11,478–11,482.
27. Wang, B., Ugen, K. E., Srikantan, V. et al. (1993) Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90**, 4156–4160.
28. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J. and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
29. Liang, H., Nishioka, Y., Reich, C. F., Pisetsky, D. S. and Lipsky, P. E. (1996) Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**, 1119–1129.
30. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
31. Hacker, H., Mischak, H., Miethke, T., et al. (1998) CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* **17**, 6230–6240.
32. Macfarlane, D. E. and Manzel, L. (1998) Antagonism of immunostimulatory CpG-oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. *J. Immunol.* **160**, 1122–1131.
33. Yi, A. K., and Krieg, A. M. (1998) Rapid induction of mitogen-activated protein kinases by immune stimulatory CpG DNA. *J. Immunol.* **161**, 4493–4497.
34. Heeg, K. and Zimmermann, S. (2000) CpG DNA as a Th1 trigger. *Int. Arch. Allergy Immunol.* **121**, 87–97.
35. Yi, A. K. and Krieg, A. M. (1998) CpG DNA rescue from anti-IgM-induced WEHI-231 B lymphoma apoptosis via modulation of I kappa B alpha and I kappa B beta and sustained activation of nuclear factor-kappa B/c-Rel. *J. Immunol.* **160**, 1240–1245.
36. Schnare, M., Holt, A. C., Takeda, K., Akira, S., and Medzhitov, R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. *Curr. Biol.* **10**, 1139–1142.
37. Chace, J. H., Hooker, N. A., Mildestein, K. L., Krieg, A. M. and Cowdery, J. S. (1997) Bacterial DNA-induced NK cell IFN-gamma production is dependent on macrophage secretion of IL-12. *Clin. Immunol. Immunopathol.* **84**, 185–193.
38. Tascon, R. E., S. Ragno, D. B. Lowrie, and M. J. Colston. (2000) Immunostimulatory bacterial DNA sequences activate dendritic cells and promote priming and differentiation of CD8+ T cells. *Immunology* **99**, 1–7.
39. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D. and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-

- gamma production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* **29**, 2344–2353.
40. Beasley, R., Crane, J., Lai, C. K. and Pearce, N. (2000) Prevalence and etiology of asthma. *J. Allergy Clin. Immunol.* **105**, S466–S472.
 41. von Mutius, E., Fritzsche, C., Weiland, S. K., Roll, G. and Magnussen, H. (1992) Prevalence of asthma and allergic disorders among children in united Germany: a descriptive comparison. *Bmj* **305**, 1395–1399.
 42. von Mutius, E., Martinez, F. D., Fritzsche, C., Nicolai, T., Reitmeir, P. and Thiemann, H. H. (1994) Skin test reactivity and number of siblings. *Bmj* **308**, 692–695.
 43. Shaheen, S. O., Aaby, P., Hall, A. J., et al. (1996) Measles and atopy in Guinea-Bissau. *Lancet* **347**, 1792–1796.
 44. Shirakawa, T., Enomoto, T., Shimazu, S. and Hopkin, J. M. (1997) The inverse association between tuberculin responses and atopic disorder. *Science* **275**, 77–79.
 45. Kline, J. N., Waldschmidt, T. J., Businga, T. R., et al. (1998) Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555–2559.
 46. Kline, J. N. (2000) Effects of CpG DNA on Th1/Th2 balance in asthma. *Curr. Top Microbiol. Immunol.* **247**, 211–225.
 47. Broide, D., Schwarze, J., Tighe, H. et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054–7062.
 48. Sur, S., Wild, J. S., Choudhury, B. K., Sur, N., Alam, R. and Klinman, D. M. (1999) Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* **162**, 6284–6293.
 49. Shirota, H., Sano, K., Kikuchi, T., Tamura, G. and Shirato, K. (2000) Regulation of T-helper type 2 cell and airway eosinophilia by transmucosal coadministration of antigen and oligodeoxynucleotides containing CpG motifs. *Am. J. Respir. Cell Mol. Biol.* **22**, 176–182.
 50. Serebrisky, D., Teper, A. A., Huang, C. K., et al. (2000) CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J. Immunol.* **165**, 5906–5912.
 51. Iwamoto, I., Nakajima, H., Endo, H. and Yoshida, S. (1993) Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. *J. Exp. Med.* **177**, 573–576.
 52. Gavett, S. H., O'Hearn, D. J., Li, X., Huang, S. K., Finkelman, F. D. and Wills-Karp, M. (1995) Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J. Exp. Med.* **182**, 1527–1536.
 53. Kline, J. N., Krieg, A. M., Waldschmidt, T. J., Ballas, Z. K., Jain, V. and Businga, T. R. (1999) CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J. Allergy Clin. Immunol.* **104**, 1258–1264.
 54. Chiaramonte, M. G., Hesse, M., Cheever, A. W. and Wynn, T. A. (2000) CpG oligonucleotides can prophylactically immunize against Th2-mediated schis-

- tosome egg-induced pathology by an IL-12-independent mechanism. *J. Immunol.* **164**, 973–985.
55. Agranovich, I., Scott, D. E., Terle, D., Lee, K. and Golding, B. (1999) Down-regulation of Th2 responses by *Brucella abortus*, a strong Th1 stimulus, correlates with alterations in the B7.2-CD28 pathway. *Infect. Immun.* **67**, 4418–4426.
56. Meissner, N., Kochs, S., Coutelle, J., et al. (1999) Modified T-cell activation pattern during specific immunotherapy (SIT) in cat-allergic patients. *Clin. Exp. Allergy* **29**, 618–625.
57. O'Brien, R. M., Xu, H., Rolland, J. M., Byron, K. A. and Thomas, W. R. (2000) Allergen-specific production of interferon-gamma by peripheral blood mononuclear cells and CD8 T cells in allergic disease and following immunotherapy. *Clin. Exp. Allergy* **30**, 333–340.
58. Hansen, G., Berry, G., DeKruyff, R. H. and Umetsu, D. T. (1999) Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* **103**, 175–183.
59. Randolph, D. A., Stephens, R., Carruthers, C. J. and Chaplin, D. D. (1999) Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J. Clin. Invest.* **104**, 1021–1029.
60. Wild, J. S., Sigounas, A., Sur, N., et al. (2000) IFN-gamma-inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. *J. Immunol.* **164**, 2701–2710.
61. Sun, S., Zhang, X., Tough, D. F., and Sprent, J. (1998) Type I interferon-mediated stimulation of T cells by CpG DNA. *J. Exp. Med.* **188**, 2335–2342.
62. Zuany-Amorim, C., Haile, S., Leduc, D., et al. (1995) Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. *J. Clin. Invest.* **95**, 2644–2651.
63. Nakajima, H., Nakao, A., Watanabe, Y., Yoshida, S. and Iwamoto, I. (1994) IFN-alpha inhibits antigen-induced eosinophil and CD4+ T cell recruitment into tissue. *J. Immunol.* **153**, 1264–1270.
64. Bellinghausen, I., Metz, G., Enk, A. H., Christmann, S., Knop, J. and Saloga, J. (1997) Insect venom immunotherapy induces interleukin-10 production and a Th2- to-Th1 shift, and changes surface marker expression in venom-allergic subjects. *Eur. J. Immunol.* **27**, 1131–1139.
65. Akdis, C. A., Blesken, T., Akdis, M., Wuthrich, B. and Blaser, K. (1998) Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* **102**, 98–106.
66. Haneda, K., Sano, K., Tamura, G., et al. (1999) Transforming growth factor-beta secreted from CD4(+) T cells ameliorates antigen-induced eosinophilic inflammation. A novel high-dose tolerance in the trachea. *Am. J. Respir. Cell Mol. Biol.* **21**, 268–274.
67. Hartmann, G., Weiner, G. J. and Krieg, A. M. (1999) CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* **96**, 9305–9310.
68. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.

69. Behboudi, S., Chao, D., Klenerman, P. and Austyn, J. (2000) The effects of DNA containing CpG motif on dendritic cells. *Immunology* **99**, 361–366.
70. Bliss, J., Van Cleave, V., Murray, K., et al. (1996) IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J. Immunol.* **156**, 887–894.
71. Abramson, M. J., Puy, R. M. and Weiner, J. M. (1995) Is allergen immunotherapy effective in asthma? A meta-analysis of randomized controlled trials. *Am. J. Respir. Crit. Care Med.* **151**, 969–974.
72. Creticos, P. S., Reed, C. E., Norman, P. S., et al. (1996) Ragweed immunotherapy in adult asthma. *N. Engl. J. Med.* **334**, 501–506.
73. Mueller, H. L., and Lanz, M. (1969) Hyposensitization with bacterial vaccine in infectious asthma. A double-blind study and a longitudinal study. *Jama* **208**, 1379–1383.

Modulation of Allergic Conjunctivitis by Immunostimulatory DNA Sequence Oligonucleotides

Andrea Keane-Myers and Chi-Chao Chan

1. INTRODUCTION

Allergic eye diseases are a group of inflammatory conjunctival conditions with certain shared characteristics, including symptoms of itching and watery discharge, an increased frequency of atopy, and papillae in the conjunctiva (1). Allergic conjunctivitis is the most common allergic disease in the United States affecting over 40 million people per year (2). Patients with allergic conjunctivitis (AC) experience itching and burning sensations in the eye in response to allergens that are innocuous for normal individuals. Patients show clinical signs of itching, chemosis (conjunctival swelling), epiphora (tearing), mild conjunctival hyperemia, and lid edema (3). Current treatment regimens are grossly inadequate, spurring the intensive search for new and more effective drugs.

The conjunctiva is a semitransparent mucous membrane that covers the posterior surface of the eyelids and the anterior portion of the eyeball. It is composed of the epithelium and the substantia propria. The conjunctival epithelium is 2–5 squamous cell layers thick, and contains goblet cells and Langerhans cells, as well as a few lymphocytes and melanocytes (1). The substantia propria is a thin layer of loose connective tissue between the epithelium and sclera containing capillaries, and nerves, as well as some lymphocytes, resident mast cells, and macrophages. Conjunctival epithelial cells bind to the surface of the eye by tight junctions, thus allowing the conjunctiva to act as a barrier to most environmental insults. Although the conjunctiva forms a natural barrier to invasion by many exogenous substances, it can be penetrated by environmental allergens such as animal dander and short ragweed (RW) pollen. When such airborne allergens land on the con-

junctival surface of sensitized individuals, allergen elution and diffusion occur (4). The majority of clinical symptoms are immediate, and are caused by the degranulation of mast cells, and the agents they release into the conjunctiva. Six to 24 h after allergen challenge, a late phase response is observed, characterized by a significant infiltration of inflammatory cells including eosinophils, into the substantia propria of the conjunctiva. The eosinophil infiltration seen in the late-phase response correlates with an increase in the Th2-associated cytokines IL-4, IL-5, and IL-10 (5). Further in vivo evidence delineates the importance of Th2-associated cytokines in allergic eye diseases, as allergen sensitization significantly increases serum levels of the Th2 -dependent antibodies IgE and allergen-reactive IgG1 (6).

The human cost of eye allergies is assessed directly in terms of medication needs, sleep deficit, impaired work, and learning ability (7). Similar to respiratory-related allergic disease, the standard therapy of seasonal allergic conjunctivitis in humans includes antihistamines, mast-cell stabilizing agents, and corticosteroids. However antihistamines, as well as corticosteroids, are only symptomatic treatments, and corticosteroids may cause substantial ocular side effects, including an increase in intraocular pressure leading to the development of glaucoma and cataracts (8). In addition, mast cell stabilizing agents are not always effective, especially if symptoms are already present. Finally, conventional treatment does not eliminate the complex immune response initiating the symptoms, and disease recurs as soon as the therapy is discontinued. Therefore, there is considerable interest among ophthalmologists and pharmaceutical companies in the development of new anti-allergic and antiinflammatory compounds that can be used safely in the eye. The frequency of allergic forms of conjunctivitis and the hazards associated with topical steroids have led to the need for these alternatives including the use of immunotherapy.

Conventional immunotherapy through desensitization by repeated exposure has achieved some success, although the majority of patients remain unresponsive. Recent efforts have focused on ways to redirect the immune system from the allergy-inducing Th2 response to a protective Th1 response through the use of such compounds as immunostimulatory sequence oligodeoxynucleotides (ISS-ODN). The ability of noncoding DNA sequences to stimulate the innate immune system was suggested more than a decade ago by Yamamoto et al., who discovered that DNA purified from mycobacteria induced Interferon (α , β , and γ) production and natural killer (NK) cell activation (9). Fractionation of the mycobacterial DNA, led to the isolation of several short DNA sequences containing CpG dinucleotide cores that mediated the immunostimulatory activity. Subsequent experiments have

shown that oligonucleotides containing ISS-ODN, induce the release of IFN- α/β , IL-6, IL-12, and IL-18 primarily from monocytes and IFN- γ from NK cells (10). The immune response to ISS-ODN is similar to the innate response against intracellular pathogens, triggering the release of cytokines that bias the immune response toward development of an antigen-specific Th1 effector and memory response. It has been shown previously that immunostimulatory DNA sequences (ISS or CpG motifs) can inhibit on-going Th2/allergic response, and induce a *de novo* Th1 response in a mouse model of asthma (11). This effect was shown to be dependent on an induction of antigen-specific IFN- γ , a Th1 cytokine known to have an inhibitory effect on the development of allergic responses. In this chapter we review findings using ISS-ODN to treat allergic eye disease, in particular, allergic conjunctivitis.

2. RESULTS

To better understand the pathology of allergic eye responses, we have developed a mouse model of allergic conjunctivitis in response to short ragweed pollen (SRW), the major cause of late summer rhinoconjunctivitis in North America (5). Using this model, we observed an immediate, or early phase response, distinguished by significant increases in the clinical signs of allergic conjunctivitis, including an increase in photosensitivity, irritation, chemosis, and discharge (Fig. 1). These clinical symptoms correlated well with the degranulation of mast cells seen in histological specimens taken 1 h postallergen challenge from these same animals. Twenty-four hours after allergen challenge, a late-phase response was observed, characterized by a significant infiltration of inflammatory cells of neutrophils, macrophages, and eosinophils into the substantia propria of the conjunctiva (Fig. 1). This late-phase response correlated with an increase in the Th2-associated cytokines IL-4, IL-5, and IL-10. In addition, allergen sensitization significantly increased serum levels of the Th2-dependent antibodies IgE, and allergen-reactive IgG1. This model mimics the kinetic, physiologic, and cellular responses observed in patients with AC, and has enabled us to carefully study disease pathogenesis and evaluate new therapies such as ISS-ODN for the treatment of allergic conjunctivitis.

In a recent study, we investigated the ability of ISS-ODN to modulate allergic responses in the RW-induced mouse model of seasonal allergic conjunctivitis (SAC) (12). In this model, which was partially adopted by Miyazaki et al. (13), we induce allergic disease by sensitizing SWR/J mice with ragweed pollen conjugated with aluminum hydroxide (50 μ g RW/5mg alum) and injected into the hind footpad. Ten days after immunization, con-

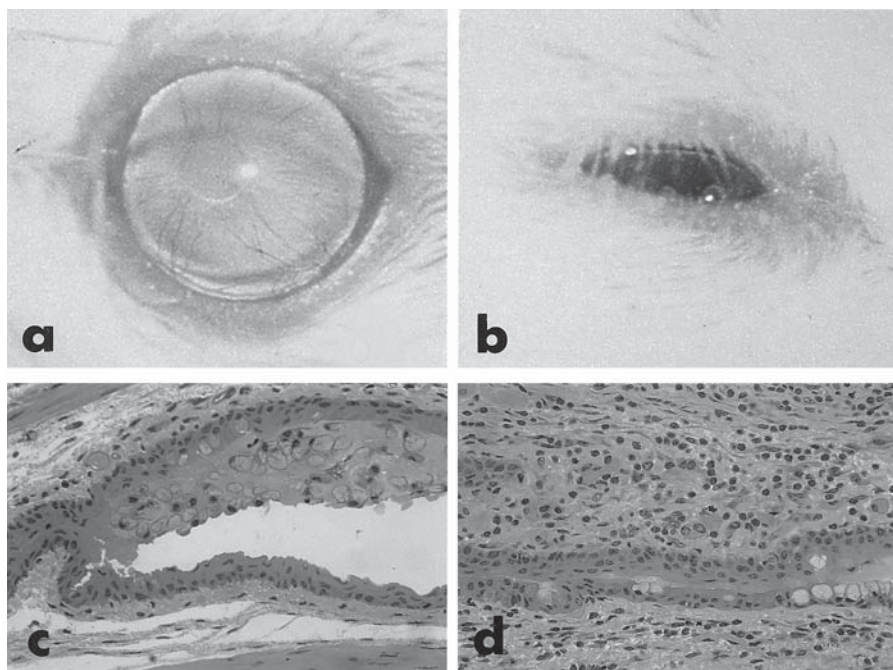


Fig. 1. Clinical (a and b) and histological findings (c and d) in our mouse model of allergic conjunctivitis. There is a significant increase of the clinical signs of allergic conjunctivitis, including an increase in photosensitivity, irritation, chemosis, and discharge within 20 min after RW challenge in RW sensitized mice (b) compared with PBS-sensitized control animals (a). Twenty-four hours after allergen challenge, the animals were sacrificed, eyes and attached lids removed, and tissue were sectioned and examined for cellular infiltrates. Histological sections of the conjunctiva reveal a late-phase response in RW sensitized animals (d), characterized by a significant infiltration of inflammatory cells into the substantia propria, compared with PBS-sensitized control animals (c). (c and d, hematoxylin and eosin, original magnification, $\times 400$)

conjunctivitis is induced by topical application (eye drops) of 2 mg RW pollen suspended in 5 μ L phosphate buffered saline (PBS) as described previously (5). Immediate responses are characterized by chemosis, conjunctival injection, and lid edema. Eyes are examined clinically for signs of an immediate hypersensitivity response 20 min after topical ragweed challenge, and the immediate hypersensitivity scores in the different groups are evaluated compared to the eyes of control animals (Fig. 1).

Table 1

Experiment	Group	IFN- γ (pg/mL)
1	ISS-ODN	4620
	Control-ISS	1230
2	ISS-ODN	6100
	Control-ISS	1350
3	ISS-ODN	7230
	Control-ISS	900
4	ISS-ODN	8200
	Control-ISS	750

In vitro IFN- γ production by RW-stimulated lymphocytes from regional lymph nodes measured with IFN- γ levels in supernatants of Amb a1 stimulated LN-derived lymphocytes in four independent experiments. The levels of IFN- γ in the ISS-ODN treated mice were always several-fold higher than the levels detected for the control-ISS treated mice. The increase in antigen-specific IFN- γ release could be neutralized by anti-IL-12 antibody administration. Stimulation of these cultures with control antigen (ovalbumin) resulted only in background levels of IFN- γ .

The authors initial studies were designed to determine whether systemic or topical administration of ISS-ODN could modify the early or late phase of allergic conjunctivitis. Mice treated systemically with ISS-ODN three d prior to RW challenge had significantly lower immediate hypersensitivity scores than control mice given either mutated, control ISS or PBS (12). Mice given ISS-ODN treatment simultaneously with the allergen challenge, also had a lower clinical score compared to control animals, although it did not reach statistical significance. Both of these treatment regimens (3 d prior and simultaneous) resulted in significant reductions in polymorphous neutrophil (PMN) and eosinophil infiltration into the conjunctiva, suggesting that ISS-ODN was capable of downregulating the late phase allergic reaction in these mice. This reduction in clinical symptoms and cellular infiltration correlated with a significant increase in IFN- γ production in ragweed-specific in vitro recall assays (Table 1), although the levels of IFN- γ produced were much higher in animals given treatment three days prior as opposed to simultaneous treatment. These findings correlate well with our data that have shown that administration of IFN- γ , either through direct injection or through DNA vaccines to be protective in either the prevention or the reversal of the allergic phenotype by redirecting the response to an antigen-specific Th1 response (Keane-Myers, manuscript in preparation). A role for the importance of IFN- γ production was also inferred by our studies using anti-IL-12

Table 2

	IgG1 (U/mL)	IgG2 (U/mL)	IgE (U/mL)
Prebleed	< 100	< 100	< 100
Day 8	377	354	< 10
Day 14	10690	1622	180
Day 18 (ISS)	12651	5040	24
Day 18 (control ISS)	16737	747	92
Day 18 PBS	22246	1040	122

Analysis of RW specific IgG1, G2a, and IgE. Mouse sera were analyzed for IgG1 and IgG2a anti-Amba1 antibodies by ELISA. Microtiter plates were coated overnight at 4°C with 5 µg Amba1/mL in carbonate buffer pH 9.0, washed with PBS/0.5% Tween-20, and nonspecific binding sites were blocked with PBS/1% bovine serum albumin (BSA). After washing, serum samples were added to duplicate wells at a 1:100 dilution in PBS/BSA, followed by serial three-fold dilutions. After overnight incubation at 4°C, the plates were washed and incubated with either alkaline phosphatase, labeled goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) diluted in PBS/BSA for 1 h, at room temperature. The plates were then washed, a solution of *p*-nitrophenyl phosphate (1 mg/mL, Boehringer Mannheim, Indianapolis, IN) was added, and the colorimetric change was measured after 1 h. Each plate contained a titration of a previously quantitated serum to generate a standard curve. The standard was arbitrarily assigned a unit value, which was calculated as the reciprocal of the highest dilution of serum, which gave an OD reading of double the background. Titers of the test sera were calculated relative to this control.

neutralizing antibodies that abolished both IFN-γ production and the protective effects induced by ISS-ODN administration (12).

To minimize dosage and possible systemic side effects, drug therapy should ideally be given at the site of the target organ. Therefore, in a second series of experiments, we administered our therapy directly to the eye. In our hands, topical treatment three days prior to allergen challenge significantly reduced the immediate clinical symptoms compared to treatment with control ISS-ODN or PBS, albeit not as strongly as what was observed with systemic administration (12). Topical administration prior to challenge also significantly reduced PMN numbers in these animals, but did not significantly reduce eosinophil numbers. Also noticed was a significant increase in RW-specific IgE after day 8 postimmunization that was increased significantly by d 14 (Table 2). In a later study, Miyazaki et al. presented similar results (13, personal communication).

An increasing number of studies have found that ISS-ODN treatment is highly sensitive to both the dosage and schedule of the regimen and the

concentration of the compound, and if given excessively, or at too high a concentration, ISS-ODN can actually strongly exacerbate disease (14,15). This dosing problem could potentially be compounded with the use of eye drops, as patients are known to use topical administration of eye drops more freely than other dosing regimens. In fact, this potential complication of ISS-ODN treatment suggests that pharmaceutical companies and physicians must remind their patients to adhere strictly to his/her prescription for the dosage and regimen of appropriate applications.

To induce a two-phase allergic response, a sensitization and a provocation stage must occur. Since both of these phases must occur prior to the development of clinical symptoms, practitioners only see patients after allergic disease has already been established. In the case of allergic responses to environmental allergens such as ragweed, patients have likely been challenged multiple times over a period of months. Therefore, we were interested in developing an ISS-ODN treatment regimen whereby we could specifically redirect an already developed Th2 response to a RW-specific Th1 response. We were also interested in evaluating if such a redirection could protect the animal from developing allergic inflammation upon subsequent RW challenge. To evaluate the effects of ISS-ODN on established RW-induced allergic conjunctivitis we sensitized mice with RW, and challenged with RW-containing eye drops 10 d later. The mice were systemically treated with ISS-ODN or control ISS on d 16 postimmunization and rechallenged on d 17. Throughout the experiment, mouse sera were analyzed for IgG1, IgG2a, and IgE anti-Amb a1 antibodies by ELISA. In RW-sensitized and -challenged animals there was a striking increase in antigen specific IgG1 and IgE levels over what was seen with control animals (Table 3). Systemic administration of ISS-ODN prior to rechallenge, significantly reduced the levels of antigen specific IgE and increased the levels of IgG2a, with no significant effect on IgG1 levels. ISS-ODN treatment after the initial RW sensitization and challenge also inhibited the late-phase cellular infiltration and induced RW-specific Th1 response (Fig. 2) to a subsequent challenge.

3. DISCUSSION

Why does ISS-ODN inhibit ocular allergic inflammation? Recognition of ISS motifs appears to have evolved in order to defend against invading intracellular pathogens. In this respect, it appears that ISS provides a danger/alarm-like signal required for this vital Th1 immune response. It has been suggested that the significant increase in asthma and allergies may actually be a side effect of progress in the area of infection control. If bacte-

Table 3

	Treatment	Mean	S.E.	P
No. of PMN	ISS-ODN	4.0	1.4	
	Control-ISS	59.1	33.3	0.0001
	PBS	36.5	5.1	0.01
No. of eosinophil	ISS-ODN	2.7	1.3	
	Control-ISS	11.3	2.9	0.007
	PBS	7.8	1.5	0.16

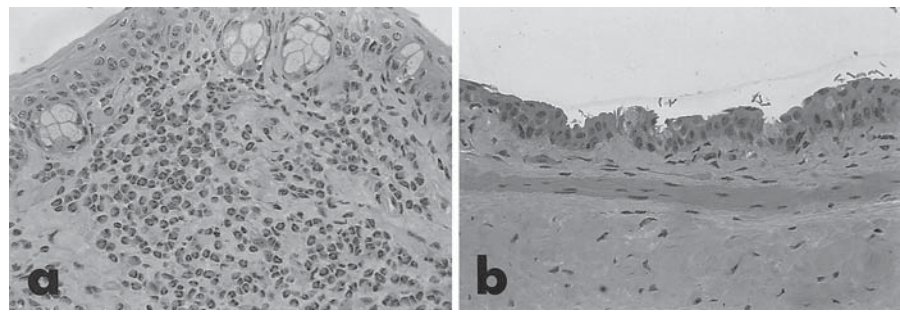


Fig. 2. To evaluate the effects of ISS-ODN on established RW-induced allergic conjunctivitis we sensitized mice with RW, and challenged with RW- containing eye drops 10 d later. The mice were systemically treated with ISS-ODN, or control ISS on day 16 postimmunization and rechallenged on d 17. ISS-ODN treatment after the initial RW sensitization and challenge (b), inhibited the late-phase cellular infiltration (a) to a subsequent challenge (hematoxylin and eosin, original magnification, $\times 400$).

rial products, including bacterial DNA, induce a shift toward a Th1 response, the lack of exposure to such products throughout life could conceivably result in a shift in the immune system, at the population level, toward Th2 type responses. We now have evidence to suggest that the administration of ISS-ODN can be used to shift the Th1/Th2 balance in experimental animal models of allergy. In our hands, ISS-ODN administration was more effective than dexamethasone in inhibiting the allergic response. Mechanistically, the ISS-ODN-induced immunomodulatory effects were abolished when mice were treated with anti-IL-12 neutralizing antibodies, suggesting a pivotal role for type 1 cytokines in the inhibition of both the immediate hypersensitivity and the late-phase cellular infiltration.

In vitro studies have found ISS-ODN induces IL-12, TNF- α , IL-6, IL-1 β , IL-1ra, MIP-1 β , MCP-1, IFN α , β , and γ , and IL-18 (16). We are in the process of using ISS treatment in various cytokine knockout and transgenic mice to determine the role of ISS on amelioration of the allergic response in vivo. There is little knowledge concerning the mechanisms by which these genes are activated by ISS-ODN. In the case of conjunctival allergy, virtually no data have been reported. Although both the NF- κ B and the MAPK pathways appear to be important in signaling responses to ISS-ODN, further details of ISS-ODN signaling pathways have been difficult to elucidate (17, 18). A major obstacle to finding such signaling pathways is that to transmit signals, the cell must internalize ISS-ODN, and to date, no internal CpG receptors have been found. Recently, Raz and colleagues elegantly demonstrated that ISS-ODN activates DNA-dependent protein kinase (DNA-PK), which can phosphorylate p53 in vitro, leading to increased transcriptional activation of genes involved in cell cycle arrest. DNA-PK activation can phosphorylate the IKK and cause NF- κ B activation (19).

In addition to its important role in cytokine production and subsequent cellular inflammation, ISS-ODN treatment also appears to effect the immediate allergic response. This immediate hypersensitivity reaction is initiated by mast cell degranulation and the subsequent release of mediators and cytokines into the tissue. We have observed some reduction in clinical symptoms to allergen challenge with simultaneous treatment protocol, suggesting that ISS-ODN may be affecting mast cell stability. We are in the process of directly examining the effects of ISS-ODN on mast cell stability by treating animals challenged with compound 48/80, a chemical that induces the degranulation of mucosal mast cells and subsequent release of inflammatory mediators (20).

In summary, the results of ISS-ODN therapy presented here provide an alternative medication to the current practice of allergen protein desensitization for allergic eye disease that has relatively low efficacy and high potential for significant side effects including anaphylaxis. Pre-priming with ISS-ODN via the systemic or local ophthalmic or conjunctival route in allergic patients may inhibit the allergic inflammatory response in allergic patients after allergen exposure.

We are planning to begin some pilot studies using ISS treatment in clinical trials on patients with seasonal allergic conjunctivitis in the near future. We must first assess the safety and efficacy of topical ISS treatment for patients with severe ocular allergy. We believe that ISS therapy could significantly decrease inflammation without any of the resultant adverse effects associated with corticosteroid treatment including glaucoma and cataract

formation. We also hope that ISS treatment would be without the adverse effect of irritation induced by the mast-cell stabilizer chromolyn sodium. ISS might be free from the rebound effects often seen with such over-the-counter preparations such as Vasocon, which remove redness from the eyes by constricting conjunctival capillaries, but do not significantly effect inflammation levels. In addition, rather than being a mere palliative treatment, ISS treatment should lead to long-term protection from allergic ocular disease. The future application of ISS-ODN therapy to human allergic diseases will depend on how well the inhibitory effects of ISS-ODN in murine models of experimental allergy translate to related human diseases.

REFERENCES

1. Hingorani, M, C. V., Buckley, R. J., and Lightman, S. L. (1998) The role of conjunctival epithelial cells in chronic ocular allergic disease. *Exp. Eye Res.* **67**, 491–500.
2. Friedlaender, M. H. (1993) Conjunctivitis of allergic origin: clinical presentation and differential diagnosis. *Surv. Ophthalmol* **38**, 105–114.
3. Liu, G., K.-M. A., Miyazaki, D., Tai, A., Ono, S. J. (1999) Molecular and cellular aspects of allergic conjunctivitis. *Chem Immunol.* **73**, 39–58.
4. Naclerio, R. and Solomon, W. (1997). Rhinitis and Inhalant Antigens. *JAMA* **278**, 1842–1848.
5. Magone, M. T., Chan, C.-C., Rizzo, L. V., Kozhich, A. T., and Whitcup, S. (1998) A Novel Murine Model of allergic Conjunctivitis. *Clinical Immunol. Immunopathology* **87**, 75–84.
6. Keane-Myers, A. M., Miyazaki, D., Liu, G., Dekaris, I., Ono, S., and Dana, M. R. (1999) Prevention of allergic eye disease by treatment with IL-1 receptor antagonist. *Invest. Ophthalmol. Vis. Sci.* **40**, 3041–3046.
7. Weinstein, A. G., McKee, L., Stapleford, J., and Faust, D. (1996) An economic evaluation of short-term inpatient rehabilitation for children with severe asthma. *J. Allergy Clin. Immunol.* **98**, 264–273.
8. Bonini, S., Lambiase, A., and Juhas, T. (1999) Allergic conjunctivitis. *Dev. Ophthalmol.* **30**, 54–61.
9. Tokunaga, T., Yamamoto, T. and Yamamoto, S. (1999) How BCG led to the discovery of immunostimulatory DNA. *Jpn. J. Infect. Dis.* **52**, 1–11.
10. Van Uden, J. and Raz, E. (1999) Immunostimulatory DNA and applications to allergic disease. *J. Allergy Clin. Immunol.* **104**, 902–910.
11. Kohama, Y., Akizuki, O., Hagihara, K., Yamada, E., and Yamamoto, H. (1999) Immunostimulatory oligodeoxynucleotide induces TH1 immune response and inhibition of IgE antibody production to cedar pollen allergens in mice. *J. Allergy Clin. Immunol.* **104**, 1231–1238.
12. Magone, M. T., Chan, C. C., Beck, L., Whitcup, S. M. and Raz, E. (2000) Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. *Eur. J. Immunol.* **30**, 1841–1850.

13. Miyazaki, D., Liu, G., Clark, L., and Ono, S. J. (2000) Prevention of acute allergic conjunctivitis and late-phase inflammation with immunostimulatory DNA sequences. *Invest. Ophthalmol. Vis. Sci.* **41**, 3850–3855.
14. Broide, D. and Raz, E. (1999). DNA-Based immunization for asthma. *Int. Arch. Allergy Immunol.* **118**, 453–456.
15. Broide, D., Schwarze, J., Tighe, H., et al. (1998) Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* **161**, 7054–7062.
16. Goodman, J. S., Van Uden, J. H., Kobayashi, H., Broide, D., and Raz, E. (1998). DNA immunotherapeutics: new potential treatment modalities for allergic disease. *Int. Arch. Allergy Immunol.* **116**, 177–187.
17. Raz, E. and Spiegelberg, H. L. (1999) Deviation of the allergic IgE to an IgG response by gene immunotherapy. *Int. Rev. Immunol.* **18**, 271–289.
18. Kobayashi, H., Horner, A. A., Takabayashi, K., et al. (1999). Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity. *Cell Immunol* **198**, 69–75.
19. Chu, W.-M., Gong, X., Li, Z.-W., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
20. Li, Q., Luyo, D., Hikita, N., Whitcup, S. M. and Chan, C. C. (1996) Compound 48/80-induced conjunctivitis in the mouse: kinetics, susceptibility, and mechanism. *Int. Arch. Allergy Immunol.* **109**, 277–285

PART VII
APPLICATIONS—CANCER

CpG Oligodeoxynucleotides and Monoclonal Antibody Therapy of Lymphoma

Thomas L. Warren and George J. Weiner

1. INTRODUCTION

In the 1890's, Dr. William Coley reported on the use of heat killed bacterial extracts (Coley's toxin) to treat malignancy (1,2). His work, although promising, was associated with significant toxicity, and was difficult to reproduce. Additional attempts in the 20th century at immunotherapy of cancer involving nonspecific stimulation of the immune system were based on the hope that a general increase in immune reactivity would lead to an anti-tumor response. In 1976, the bacillus Calmette-Guérin (BCG) was found to be effective at eliminating local tumors and preventing tumor recurrences in patients with superficial bladder cancer. BCG is now accepted as a treatment of bladder cancer (3). The efficacy of such immunotherapeutic approaches has been attributed to bacterial cell wall components, such as endotoxin. There is now rapidly accumulating evidence that other bacterial products, including the bacterial DNA itself, also have immunostimulatory effects.

Indeed, we now know that bacterial DNA, but not vertebrate DNA, can activate B cells, natural killer (NK) cells, and macrophages (reviewed in Part III). Synthetic oligodeoxynucleotides containing unmethylated cytosine-guanine motifs on a DNA nuclease-resistant phosphorothioate backbone (CpG-ODN) can have similar effects. (4-10). In addition to inducing cellular proliferation and a change in phenotype, CpG-ODN can induce the production of a wide variety of cytokines including IL-6, IL-10, IL-12, IL-18, IFN- α , IFN- γ , and TNF- α (4-16). These immunostimulatory effects are owing to motifs that include unmethylated cytosine-guanine (C-G) dinucleotides (6). Vertebrate DNA contains a lower frequency of unmethylated C-G dinucleotides owing to both the under representation of C-G dinucleotide

(termed C-G suppression) and the methylation of 80% of the C-G dinucleotides (17). The immunostimulatory effects of the CpG motif itself vary depending on the surrounding bases. In addition, the effects of specific CpG ODN vary from cell type to cell type and species to species (9). Ongoing investigations are exploring the molecular signaling responsible for these effects, with recent studies suggesting TLR9 plays a central role (18).

Irrespective of the molecular mechanisms involved, the effects of CpG ODN on cellular function and cytokine production have led to evaluation of CpG ODN as antitumor agents in a variety of animal models with promising results. CpG ODN has direct anti-tumor effects on NK cell sensitive tumors (9,19), and can also function as potent immune adjuvants when coadministered with soluble antigen. CpG ODN is as effective as complete Freund's adjuvant at enhancing production of antitumor antibody and protecting from a tumor challenge following immunization (20–22). CpG ODN can also enhance induction of an antigen specific cytotoxic T cell (CTL) response when used as either an adjuvant in peptide immunization or with dendritic cell immunization (15,23–25). The presence of CpG motifs also appears to play a role in the immunogenicity of plasmid DNA vaccines (26,27).

2. RESULTS

Given that CpG-ODN activates many of the cells thought to participate in antibody dependent cellular cytotoxicity (ADCC), we explored whether the addition of CpG ODN to anti-tumor moAb could enhance the anti-tumor effects of the antibody (28,29). Indeed, we found this to be the case. These studies utilized the 38C13 murine B-cell lymphoma model that has been used extensively in studies of antibody based therapy (30–35). The idiotype of the 38C13 surface IgM serves as the target tumor antigen (36,37). The addition of CpG ODN to anti-idiotypic (i.e., antitumor) moAb markedly enhanced the anti-tumor effect of moAb (Table 1) (29). CpG ODN itself had no significant anti-tumor activity. Antitumor moAb therapy alone had modest anti-tumor activity under these conditions and resulted in 10% long-term survival whereas the addition of CpG ODN to moAb therapy resulted in 80% long-term survival. The survival of mice treated with CpG ODN and moAb was statistically superior to that of control groups (vs CpG alone, $p < 0.001$; vs moAb therapy alone, $p = 0.01$). There was no obvious toxicity seen with CpG ODN.

CpG-ODN with a different sequence was tested and found to have similar ability to enhance the efficacy of moAb therapy. In contrast, a methylated version of CpG ODN did not impact significantly on survival. It therefore is unlikely that other mechanisms, such as antisense activity, contributed to the anti-tumor effect seen in vivo with CpG-ODN and moAb. IL-2 has been

Table 1 Summary of results evaluating the combination of CpG ODN and moAb in the 38C13 lymphoma model			
	Median survival (days)	Overall survival (%)	<i>p</i> value (overall survival)
CpG ODN enhances the efficacy of moAb therapy			
Untreated Control	18	0	
CpG 1758 Alone	24	0	
moAb Alone	27	10	
Control 1758 and moAb	28	20	<i>p</i> = 0.12 vs moAb Alone
IL-2 and moAb	35	40	<i>p</i> = 0.2 vs CpG 1758 and moAb
CpG 1758 and moAb	> 60	80	<i>p</i> < 0.001 vs CpG 1758 Alone <i>p</i> = 0.011 vs moAb Alone
Larger doses of CpG ODN are more effective when used in combination with moAb			
Untreated Control	14	0	
moAb Alone	21	0	
CpG 1826 100µg Alone	14	0	<i>p</i> = 0.135 vs Untreated Control
moAb and CpG 1826 200µg	> 60	70	<i>p</i> < 0.001 vs Untreated Control
moAb and CpG 1826 20µg	35	30	<i>p</i> < 0.001 vs Untreated Control
moAb and CpG 1826 2.0µg	25	0	<i>p</i> > 0.05 vs Untreated Control
CpG ODN is effective when administered before or after moAb			
CpG 1826 D 3 Alone	24	0	
moAb D 3 Alone	33	0	
moAb D 3 and CpG 1826 D 0	>60	70	<i>p</i> < 0.001 vs moAb D 3 Alone
moAb D 3 and CpG 1826 D 2	50	50	<i>p</i> < 0.001 vs moAb D 3 Alone
moAb D 3 and CpG 1826 D 3	59	40	<i>p</i> < 0.001 vs moAb D 3 Alone
moAb D 3 and CpG 1826 D 4	>60	70	<i>p</i> < 0.001 vs moAb D 3 Alone
moAb D 3 and CpG 1826 D 6	34	20	<i>p</i> < 0.001 vs moAb D 3 Alone
Multiple doses CpG ODN and moAb are more effective than multiple doses of moAb			
Untreated Control	17	0	
moAb Alone D 5,7,9	22	0	
moAb alone D 5,7,9,19,21,24	28	0	
moAb and CpG 1826 D 7,10,12	29	0	
moAb and CpG 1826 D 3,5,7	34	50	<i>p</i> < 0.001 vs Untreated Control
moAb and CpG 1826 D 5,7,9	48	30	<i>p</i> < 0.001 vs moAb alone D 5,7,9
moAb and CpG 1826 D 5,7,9,19,21,24	>60	60	<i>p</i> = 0.14 vs moAb and CpG 1826 D 5,7,9 <i>p</i> < 0.001 vs moAb alone D 5,7,9

shown to enhance the efficacy of anti-idiotypic moAb therapy in this model (31). CpG-ODN was more effective than IL-2 in this model, with CpG ODN plus moAb resulting in 80% long-term survival, whereas IL-2 plus moAb resulting in only 40% long-term survival (29).

We also performed a dose-response evaluation by varying the dose of CpG-ODN (28). Higher doses of CpG-ODN enhanced the anti-tumor activity of moAb more effectively than lower doses. In studies exploring the sequence of administration, mice were inoculated with tumor on d 0 with and treated with moAb on d 3. CpG-ODN was administered as a single dose on d 0, 2, 3, 4, or 6 after tumor inoculation. Administration of CpG-ODN within 2 d of moAb therapy significantly improved survival, while mice treated with CpG-ODN 4 d after moAb had survival that was indistinguishable from moAb alone. These studies demonstrate that CpG-ODN can be administered before or after moAb. The ability of CpG-ODN to enhance the response to moAb therapy is diminished when the administration of CpG-ODN is delayed more than 2 d after the administration of moAb.

We next evaluated if multiple doses of CpG-ODN and moAb could eliminate a larger tumor burden compared to single doses, or if multiple doses of moAb alone could yield results similar to those found with the combination of CpG-ODN and moAb. Multiple doses of CpG-ODN and moAb resulted in improved survival even when the therapy was delayed until seven d after tumor inoculation, and prolonged survival was noted even when therapy was begun 10 d after tumor inoculation (when tumor was palpable). No toxicity was observed even with repeated doses of moAb plus CpG-ODN. Multiple doses of moAb alone could not achieve the antitumor activity of even single doses of moAb and CpG-ODN.

It is possible direct effects of CpG-ODN on tumor cells, and not the enhanced ADCC capabilities of effector cells, could have contributed to the observed synergy between moAb and CpG-ODN. Possibilities include enhanced expression of target antigen by CpG ODN, modification of the signaling properties of the lymphoma cells by CpG-ODN, or the ability of CpG-ODN to increase the sensitivity of the tumor cells to ADCC. We found none of these effects in the 38C13 model. CpG-ODN as a single agent had no detectable effect on the growth or phenotype of 38C13 cells either *in vitro* or *in vivo*. CpG-ODN induced no detectable changes in signaling of 38C13 cells either alone, or in response to anti-Id. Pretreatment of 38C13 lymphoma cells had no impact on their sensitivity to ADCC, and also did not increase their sensitivity to complement mediated lysis (unpublished observations). Thus, in this particular model of a high grade lymphoma, the

ability of CpG-ODN to enhance the efficacy of moAb therapy appears to be owing to activation of the effector cells responsible for ADCC.

The 38C13 model has been valuable as a tool for evaluation of lymphoma immunotherapy. However, it clearly differs from human lymphomas in general, and slow growing low grade human lymphomas such as follicular lymphomas, in particular. Indeed, in contrast to the results outlined previously, CpG ODN alone does have a significant impact on the phenotype of human primary malignant B cells. Decker et al. had found that CpG ODN could alter the cytokine production and phenotype of chronic lymphocytic leukemia cells (38,39). We evaluated whether CpG-ODN has direct effects on cells harvested from patients with a variety of lymphomas (40). Single cell suspensions from fresh lymph node samples, peripheral blood and pleural fluid were stimulated with CpG ODN or control ODN. Their phenotype was then evaluated. CpG-ODN, but not control ODN, increased the surface expression of a variety of molecules (CD40, CD80, CD86, CD54) on malignant B cells that are involved in costimulation. In contrast, no alteration in phenotype of B cells was seen in cells obtained from a patient with reactive follicular hyperplasia. CpG-ODN also enhanced expression of both class I and class II MHC, and the antigen recognized by the anti-HLA-DR antibody Hu1D10.

CD20 has proven to be a valuable target for both unlabeled and radiolabeled moAbs (41,42). Importantly, CD20 expression was increased in a variety of B-cell malignancies in response to CpG-ODN. An inverse correlation was found between baseline expression of CD20 and its expression after exposure to CpG-ODN. This was most pronounced in B-CLL and marginal zone lymphoma, which are known to have relatively low baseline expression of this antigen. These findings suggest CpG-ODN could enhance reactivity of both unlabeled and radiolabeled anti-CD20 moAb with cells that usually express only low levels of CD20. Ongoing studies are evaluating whether the ability of CpG-ODN to enhance expression of CD20 also alters the sensitivity of such cells to CD20-mediated ADCC or complement mediated lysis.

3. DISCUSSION

Taken together, these studies suggest the CpG ODN could enhance the efficacy of moAb therapy by both increasing expression of the target antigen and enhancing ADCC (*see* Table 2). The combination of the anti-CD20 moAb Rituximab and CpG ODN is a particularly attractive combination. One potential concern is that CpG ODN might activate B cells and induce

Table 2
Summary of Observed Effects of CpG ODN on moAb Therapy of Lymphoma in Mice and Humans

CpG ODN and moAb in the 38C13 murine lymphoma model	CpG ODN and moAb in human lymphoma
CpG ODN enhances the efficacy of moAb therapy if given within 2 days of moAb	CpG ODN upregulates expression of a variety of antigens including CD20 by primary lymphoma and CLL cells
Repeated doses of CpG ODN plus moAb cure mice with large tumor burdens that can not be cured with other immunotherapeutic approaches	Impact of CpG ODN on CD20 expression correlates inversely with baseline expression
Synergy in the 38C13 model appears to be owing to ability of CpG ODN to enhance effector cell function	An ongoing phase I trial is exploring the effect of CpG ODN in human lymphoma

proliferation of the malignant cells, or protect them from apoptosis. Preliminary studies suggest the proliferative effect of CpG ODN is more pronounced on benign cells than on malignant cells, so this may not be a major issue (40). However, the potential stimulatory or anti-apoptotic effects of CpG ODN on malignant B cells clearly deserves further evaluation.

In conclusion, recent advances in our understanding of tumor immunology and the immune system are allowing for the development of new, rational approaches to cancer immunotherapy. The development of clinically useful moAbs directed against a variety of antigens for the treatment of human lymphomas is encouraging and exciting. The studies outlined previously indicate that CpG ODN combined with antitumor moAb therapy is an attractive approach. Clinical trials of CpG ODN in humans have recently been initiated including a phase I study on the safety and tolerability of CpG ODN in patients with relapsed or refractory B cell lymphoma. Based in part on the preclinical data presented in this report, subsequent trials will explore the potential of the combination of CpG ODN and moAb.

REFERENCES

1. Coley, W. B. (1893) The Treatment of Malignant Tumors by Repeated Inoculations of Erysipelas with a Report of Ten Original Cases. *Amer. J. Med. Sci.* **105**, 487–511.

2. Coley, W. B. (1894) Treatment of Inoperable Malignant Tumors with the Toxins of Erysipelas and the Bacillus Prodigiosus. *Amer. J. Med. Sci.* **108**, 183–212.

3. Morales, A. (1978) Adjuvant immunotherapy in superficial bladder cancer. *Natl. Cancer Inst. Monogr.* **49**, 315–319.
4. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
5. Messina, J. P., Gilkeson, G. S., and Pisetsky, D. S. (1991) Simulation of In Vitro Murine Lymphocyte Proliferation by Bacterial DNA. *J. Immunol.* **147**, 1759–1764.
6. Krieg, A. M., Yi, A. K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
7. Yamamoto, S., Yamamoto, T., Shimada, S., et al. (1992) DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* **36**, 983–997.
8. Yamamoto, S., Kuramoto, E., Shimada, T., and Tokunaga, T. (1988) In Vitro Augmentation of Natural Killer Cell Activity and Production of Interferon-Alpha/Beta and Gamma with Deoxyribonucleic Acid Fraction From Mycobacterium Bovis BCG. *Jpn. J. Cancer Res.* **79**, 866.
9. Ballas, Z. K., Rasmussen, W. L., Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840–1845.
10. Stacey, J. J., Sweet, M. J., and Hume, D. A. (1996) Macrophages Ingest and Are Activated by Bacterial DNA. *J. Immunol.* **157**, 2116–2122.
11. Redford, T. W., Yi, A. K., Ward, C. T., and Krieg, A. M. (1998) Cyclosporin A enhances IL-12 production by CpG motifs in bacterial DNA and synthetic oligodeoxynucleotides. *J. Immunol.* **161**, 3930–3935.
12. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* **29**, 2344–2353.
13. Sparwasser, T., Miethke, T., Lipford, G., et al. (1997) Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. *Eur. J. Immunol.* **27**, 1671–1679.
14. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
15. Warren, T. L., Bhatia, S. K., Acosta, A. M., et al. (2000) APC stimulated by CpG oligodeoxynucleotide enhance activation of MHC class I-restricted T cells. *J. Immunol.* **165**, 6244–6251.
16. Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999) CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA* **96**, 9305–9310.
17. Bird, A. P., Taggart, M. H., Nicholls, R. D., and Higgs, D. R. (1987) Non-methylated CpG-rich islands at the human alpha-globin locus: implications for evolution of the alpha-globin pseudogene. *EMBO J.* **6**, 999–1004.

18. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740–745.
19. Carpentier, A. F., Chen, L., Maltonti, F., and Delattre, J. Y. (1999) Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mice. *Cancer Res.* **59**, 5429–54232.
20. Weiner, G. J., Liu, H. M., Wooldridge, J. E., Dahle, C. E., and Krieg, A. M. (1997) Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. USA* **94**, 10,833–10,837.
21. Liu, H. M., Newbrough, S. E., Bhatia, S. K., Dahle, C. E., and Krieg, A. M., Weiner, G. J. (1998) Immunostimulatory CpG oligodeoxynucleotides enhance the immune response to vaccine strategies involving granulocyte-macrophage colony-stimulating factor. *Blood* **92**, 3730–3736.
22. Davis, H. L., Weeranta, R., Waldschmidt, T. J., Tygrett, L., Schorr, J., and Krieg, A. M. (1998) CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876.
23. Cho, H. J., Takabayashi, K., Cheng, P. M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nat. Biotechnol.* **18**, 509–514.
24. Roman, M., Martinorozco, E., Goodman S, et al. (1997) Immunostimulatory Dna sequences function as t helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854.
25. Vabulas, R. M., Pircher, H., Lipford, G. B., Hacker, H., and Wagner, H. (2000) CpG-DNA activates in vivo T cell epitope presenting dendritic cells to trigger protective antiviral cytotoxic T cell responses. *J. Immunol.* **164**, 2372–2378.
26. Klinman, D. M., Yamshchikov, G., and Ishigatsubo, Y. (1998) Contribution Of Cpg Motifs to the Immunogenicity Of Dna Vaccines. *J. Immunol.* **158**, 3635–3639.
27. Krieg, A. M., Yi, A. K., Schorr, J., Davis, H. L. (1998) The role of CpG dinucleotides in DNA vaccines. *Trends Microbiol.* **6**, 23–27.
28. Warren, T. L., Dahle, C. E., and Weiner, G. J. (2000) CpG oligodeoxynucleotides enhance monoclonal antibody therapy of a murine lymphoma. *Clinical lymphoma* **1**, 57–61.
29. Wooldridge, J. E., Ballas, Z., Krieg, A. M., and Weiner, G. J. (1997) Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood*, **89**, 2994–2998.
30. Starnes, C. O., Carroll, W. L., Campbell, M. J., Houston, L. L., Apell, G., and Levy, R. (1988) Heterogeneity of a murine B cell lymphoma. Isolation and characterization of idiotypic variants. *J. Immunol.* **141**, 333–339.
31. Berinstein, N. and Levy, R. (1987) Treatment of a murine B cell lymphoma with monoclonal antibodies and IL-2. *J. Immunol.* **139**, 971–976.
32. Weiner, G. J., and Kaminski, M. S. (1989) Idiotype variants emerging after anti-idiotypic monoclonal antibody therapy of a murine B cell lymphoma. *J. Immunol.* **142**, 343–351.
33. Weiner, G. J., Kaminski, M. S. (1990) Anti-idiotypic antibodies recognizing stable epitopes limit the emergence of idiotype variants in a murine B cell lymphoma. *J. Immunol.* **144**, 2436–2435.

34. Maloney, D. G., Kaminski, M. S., Burowski, D., Haimovich, J., and Levy, R. (1985) Monoclonal anti-idiotypic antibodies against the murine B cell lymphoma 38C13: characterization and use as probes for the biology of the tumor in vivo and in vitro. *Hybridoma* **4**, 191–209.
35. Kaminski, M. S., Kitamura, K., Maloney, D. G., Campbell, M. J., and Levy, R. (1986) Importance of antibody isotype in monoclonal anti-idiotypic therapy of a murine B cell lymphoma. A study of hybridoma class switch variants. *J. Immunol.* **136**, 1123–1130.
36. Eshhar, Z., Blatt, Y., Bergman, Y., and Haimovich J. (1979) Induction of secretion of IgM from cells of the B cell line 38C13 by somatic hybridization. *J. Immunol.* **122**, 2430–2434.
37. Bergman, Y. and Haimovich, J. (1977) Characterization of a carcinogen-induced murine B lymphocyte cell line of C3H/eB origin. *Eur J. Immunol.* **7**, 413–417.
38. Decker, T., Schneller, F., Sparwasser, T., et al. (2000) Immunostimulatory CpG-oligonucleotides cause proliferation, cytokine production, and an immunogenic phenotype in chronic lymphocytic leukemia B cells. *Blood* **95**, 999–1006.
39. Decker, T., Schneller, F., Kronschnabl, M., et al. (2000) Immunostimulatory CpG-oligonucleotides induce functional high affinity IL-2 receptors on B-CLL cells. Costimulation with IL-2 results in a highly immunogenic phenotype. *Exp. Hematol.* **28**, 558–568.
40. Jahrsdorfer, B., Hartmann, G., Racila, E., et al. (2001) CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens. *J. Leukoc. Biol.* **69**, 81–88.
41. McLaughlin, P., Grillo-Lopez, A. J., Link, B. K., et al. (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J. Clin. Oncol.* **16**, 2825–2833.
42. Kaminski, M. S., Zasadny, K. R., Francis, I. R., et al. (1996) Iodine-131 - Anti-B1 radioimmunotherapy for B-cell lymphoma. *J. Clin. Oncol.* **14**, 1974–1981.

PART VIII

INFLAMMATION AND AUTOIMMUNITY

The Antigenicity of Bacterial DNA

David S. Pisetsky

1. INTRODUCTION

1.1. The Dogma on Antigenicity

DNA is a complex macromolecule whose immunological properties vary with base sequence and backbone structure. Although now recognized as important for normal immunity, the antigenic and immunogenic properties of DNA were originally conceptualized entirely in the context of systemic lupus erythematosus (SLE). This prototypic autoimmune disease is characterized by the production of antibodies to DNA (anti-DNA). These antibodies occur prominently in the sera of lupus patients and serve as markers of diagnosis and prognosis. The close association of anti-DNA with SLE has suggested that elucidating this response would provide fundamental insights into the mechanisms of autoimmunity, as opposed to normal responses (1).

Until recently, three main ideas influenced thinking on the antigenic properties of DNA. The first idea is that anti-DNA production is exclusive to SLE. As demonstrated in many studies, antibodies to DNA, although common in SLE, are rarely encountered in either normal individuals or patients with other diseases. As such, anti-DNA has been viewed as virtually synonymous with autoimmunity. The second idea is that DNA itself is immunologically uniform and inert. Efforts to replicate SLE by immunization with DNA have been generally unsuccessful, distinguishing SLE from other autoimmune conditions where immunization with self antigens can provoke pathology in normal animals. The third idea is that the anti-DNA response in SLE is antigen driven. The most compelling evidence for this idea derives from studies of monoclonal anti-DNA antibodies from lupus mice. These antibodies display variable region somatic mutations associated with heightened affinity for DNA, pointing to selection by DNA itself (1–3).

Although a number of mechanisms can reconcile these ideas, the simplest explanation for antigen drive by DNA, despite its meager immunogenicity, is that patients with SLE have gross disturbances that allow a response to this molecule. These disturbances may relate to the composition of the B- and T- cell repertoires as well as signaling thresholds critical either in the establishment of tolerance or cell activation following contact with antigen. According to this scenario, an ensemble of defects—acquired or inherited—interact to allow responses to DNA, an otherwise blank molecule immunologically.

1.2. Revising the Dogma

Because of research over the last decade, key aspects of the dogma on DNA's antigenicity require revision. As shown both *in vitro* and *in vivo*, DNA, rather than being inert, has potent immunological properties. These properties result from characteristic sequence motifs called cytosine guanosine dinucleotide CpG motifs or immunostimulatory sequences (ISS) that make bacterial DNA a “danger” molecule which, like endotoxin, can activate the innate immune system. Furthermore, recent studies have shown that anti-DNA expression is a feature of normal immunity and not just SLE. Thus, sera of normal human subjects (NHS) display antibodies that target sites specific to bacterial DNA. The expression of these antibodies, which was missed for almost 40 yr, overturns the idea that DNA antigen drive is unique to SLE (4–6). Since other chapters in this book will discuss the immunostimulatory properties of bacterial DNA, this chapter will focus on the antigenic properties of bacterial DNA and their relationship to CpG motifs.

2. RESULTS

2.1. The Contribution of Sequence to Antigenicity

A key issue in understanding the antigenicity of DNA, concerns the various epitopes bound by normal and SLE anti-DNA. In the setting of SLE, antibody recognition of DNA has been dichotomized in terms of single-stranded (ss) and double-stranded (ds) DNA. This dichotomy implies that the key epitopes reside on the DNA backbone; in this context, dsDNA refers to the B DNA conformation, which is the classic Watson-Crick structure. Although anti-ssDNA can occasionally be found outside of SLE, anti-dsDNA is highly specific for this condition, leading to its designation as a criterion for disease classification (1,6).

Because of the ubiquity of the double helix among DNA, it has been generally assumed that all dsDNA are equivalently antigenic. Although anti-DNA assays do not always produce concordant results, the discrepancies

have usually been attributed to the operating characteristics of the assays rather than the antigenic structure of the DNA.

In the few studies where different DNA antigens were tested for activity by the same assay, marked variability was noted (7). These observations are consistent with the idea that base sequence and backbone structure can both influence antigenicity. Evidence for this possibility has also come from studies indicating that DNA in immune complexes differs in base composition from total chromosomal DNA (8,9).

2.2. Antigenicity of Bacterial DNA

Prompted by observations that anti-DNA may have sequence specificity, our laboratory began studies to characterize patterns of DNA recognition by SLE sera. Our initial approach involved assays with DNA from various mammalian and bacterial DNA that differed in base composition and sequence (10). The assumption of these studies was that anti-DNA antibodies can bind differentially to DNA depending on specificity for base sequence. Similar experiments had been performed to map epitopes on proteins using species variants differing in amino acid sequence.

This approach produced an unexpected result which forced reconsideration of DNA's antigenic properties. Using ssDNA as antigens in an enzyme-linked immunosorbent assay (ELISA), SLE sera showed similar levels of binding to the various DNA in the panel. This result is consistent with a preference of SLE sera for a conserved backbone determinant by the predominant antibodies. The striking finding, however, concerned the behavior of sera from NHS. Although prevailing ideas indicated that NHS sera are devoid of anti-DNA, our studies showed significant reactivity to two of the DNA in the panel, *Micrococcus lysodeikticus* (MC) and *Staphylococcus epidermidis* (SE). This reactivity was fully DNase sensitive and, in some instances, similar in magnitude to that of SLE sera. In these studies, NHS sera showed reactivity with both ss and ds bacterial DNA (10-12).

While antibodies to bacterial DNA had not been previously observed in NHS in almost 40 yr of study, their presence can be readily explained. Thus, DNA from bacteria differs from mammalian DNA in sequence. As such, this DNA can be recognized as foreign during infection or colonization, leading to the generation of antibodies to nonconserved sequences not subject to tolerance. In this conceptualization, DNA displays an epitope structure comprised of sequential and conformational determinants, with responses to sequential determinants a feature of normal immunity. Although coding sequences on bacterial DNA provide a rich array of potential epitopes, there appear to be restrictions on antigenicity since NHS dis-

play antibodies to only some bacterial DNA. Whether antigenicity depends on the structure of DNA or the nature of the contact with the host is not known.

Support for a role of bacterial DNA in anti-DNA expression comes from animal experiments. Unlike anti-DNA autoantibodies which are difficult to induce by immunization with mammalian DNA, antibodies to bacterial DNA are readily elicited in normal animals. When presented as protein complexes in complete Freund's adjuvant (CFA), *E. coli* (EC) dsDNA can induce antibodies that bind specifically to the immunizing antigen and fail to show reactivity with mammalian DNA. These antibodies display the immunoglobulin G (IgG) isotype and require the presence of T cells, perhaps reflecting the use of a protein carrier in this model. Using bacterial ssDNA as the immunogen, the induced antibodies show a broader specificity and bind mammalian as well as bacterial ssDNA. These findings confirm the immunogenicity of bacterial DNA and provide a system that replicates the serological features of NHS (13–14).

While the immunogenicity of bacterial DNA could relate to nonconserved sequences to which the host is not tolerant, the immunostimulatory properties of bacterial DNA may also contribute to this property. As is now known, bacterial DNA displays a host of immunostimulatory properties that rival those of endotoxin in potency and extent. These activities, which result from CpG motifs or ISS, include polyclonal B-cell activation and induction of cytokines such as IL-12, IFN- α/β , TNF- α and IFN- γ (4–5, 15–17). In total, these activities provide a built-in adjuvant for bacterial DNA, and may explain the magnitude of the responses induced by bacterial DNA in comparison to mammalian DNA. While ISS likely promote the immunogenicity of bacterial DNA, this role has not formally been established in animal models and thus remains speculative.

2.3. The Relationship of CpG Motifs to Antigenic Sequences

Although CpG motifs confer potent immunological properties on bacterial DNA, they do not appear to be the major epitopes bound by NHS. CpG motifs occur commonly on bacterial DNA, with their frequency varying depending on species and base composition. With CpG motifs as epitopes, sera binding would be expected to be much broader and crossreactive among bacterial DNA than that observed. Furthermore, the presence of CpG motifs on mammalian DNA, albeit in lower concentrations than in bacterial DNA, could provide the basis for at least some crossreactivity. The high specificity of NHS for certain bacterial DNA suggests therefore that nonconserved sequences other than CpG motifs are the target of reactivity (18).

At present, the DNA sequences on bacterial DNA that are antigenic are not known. These sequences, however, appear to occur variably among bacterial DNA, with each antigenic DNA showing at least one unique determinant. The nature of these determinants has been investigated by absorption experiments using a panel of bacterial DNA selected on the basis of frequent reactivity with NHS. Following absorption with a bacterial DNA on a cellulose affinity column, sera retained their reactivity with the other bacterial DNA tested. Since each of the bacterial DNA used as an absorbent produced a similar result, these findings indicate reactivity to nonconserved sites uniquely expressed by different bacterial DNA (19,20). Though consistent with highly restricted patterns of DNA binding based on species origin, these results do not exclude the presence of antibodies to sequences that may be more widely distributed among bacterial DNA; such crossreactive antibodies, however, appear to be at best a minority population.

The absorption analysis provides additional evidence that SLE sera bind conserved epitopes present on both bacterial and mammalian DNA. Thus, absorption of SLE sera with any DNA removed essentially all antibodies to DNA, either bacterial or mammalian. This result was obtained with each of the bacterial DNA tested. While indicating crossreactivity of SLE antibodies, these findings also indicate a deficit of antibodies specific for bacterial DNA in patient sera. These observations are consistent with either a major switch of antibody specificity from nonconserved determinants or selective deficiency in reactivity to foreign DNA (19–20).

2.4. Immunochemical Properties of Antibodies to Bacterial DNA

Although induction of anti-DNA in murine models is T dependent, NHS anti-DNA show immunochemical properties suggesting a different pathway of induction. With all bacterial DNA tested, SLE anti-DNA is predominantly IgG1 whereas NHS anti-DNA is predominantly IgG2 (11,18). These findings point to differences in the two responses in the mode of induction. Whereas IgG1 responses are usually viewed as T-dependent responses, IgG2 responses are more T independent, resembling the responses to bacterial carbohydrate antigens. Because both bacterial DNA and bacterial carbohydrates are large macromolecules with repeating structures, their induction may occur by similar mechanisms.

2.5. Distribution of DNA Epitopes

Despite the uncertainties in evaluating a macromolecular antigen of unknown valency, SLE anti-DNA has been viewed as a high avidity response. A comparison with NHS anti-DNA, however, provides a different

perspective. As shown with ssDNA in an ELISA, SLE anti-DNA requires a much higher concentration of MC DNA for 50% inhibition than NHS anti-DNA. The concentrations for inhibition by MC and CT DNA are similar using SLE sera, supporting the idea that SLE sera bind crossreactively to a determinant shared by mammalian and bacterial DNA. These results are consistent with a difference in avidity based on epitope specificity. Since SLE anti-DNA appear to recognize backbone determinants, charge-charge interactions are the main determinants of binding and may be lower avidity and less specific. In contrast, NHS sera recognize sequences, necessitating different binding mechanisms and higher specificity (12).

The difference between the binding of NHS and SLE to DNA extends to the size of the determinant recognized. Although the combining site of a single Fab region can accommodate only a few nucleotides, SLE anti-DNA require much larger pieces of DNA for stable binding, with the size dependent on whether the antigen is in the fluid or solid phase. In the fluid phase, stable binding of DNA by SLE anti-DNA occurs with DNA pieces 40–50 bases or longer; of note, some antisera require DNA pieces hundreds of bases long, a finding also observed with some murine monoclonal antibodies (21).

The reason that SLE antibodies require extended structures relates to the importance of monogamous or bivalent interaction. In this mode of binding, both Fab sites of an IgG must simultaneously contact determinants along an extended polynucleotide. Thus, crosslinking appears necessary for binding of DNA with autoimmune sera. With solid phase DNA, the size requirement for antigenicity is even greater. Using DNA size fractionated after restriction digestion by *Hinf*I, SLE sera need pieces of DNA several thousand bases in length for reactivity in an ELISA. In contrast, in the fluid phase, these pieces are antigenic, suggesting that, in the solid phase, DNA is constrained in mobility and unable to undergo rearrangements for bivalent interaction (22).

In contrast to SLE anti-DNA, NHS anti-DNA appears to bind bacterial DNA in a pattern less dependent on DNA size. As shown using *Hinf*I digests of DNA from *Klebsiella pneumoniae*, NHS sera can bind DNA fragments a few hundred bases in length under conditions in which NHS have dramatically reduced levels of binding in comparison to intact DNA (23). These observations are consistent with a direct interaction of antibodies with sequential epitopes by a monovalent mechanism. As a result, there is less need for conformational rearrangement of the backbone or crosslinking of sites along an extended polynucleotide chain. Although pointing to a distribution of epitopes along the bacterial chromosome, these experiments do not allow

Table 1
Properties of Antibodies to Bacterial DNA

Common in normals.
High affinity.
Bind ss and ds DNA.
IgG2 predominance.
Bind nonconserved sequences other than CpG.
Bind small and large DNA fragments.
Reduced in SLE.

determination of the number of such epitopes or the distance between them. Table 1 summarizes the properties of antibodies to bacterial DNA.

3. DISCUSSION

3.1. Antigenic Structure of DNA

As this review indicates, DNA displays a multifaceted antigenic structure comprised of both conserved and nonconserved determinants. Whereas SLE sera recognize conserved sites that appear to be conformations of the DNA backbone (both bacterial and mammalian), NHS recognize nonconserved sites that appear to be sequences on bacterial DNA. These sequences are distinct from CpG motifs, although their identity is unknown. Furthermore, since NHS recognize only certain bacterial DNA, the induction of these responses may reflect the nature of these sequences as well as the setting in which the host encounters DNA in infection or colonization.

3.2. Implications for Host Defense, Pathogenesis and Therapy

In view of CpG DNA's widespread immune activities, these findings immediately raise three questions. The first concerns the physiological role of anti-DNA in NHS and the ability to protect the host from bacterial DNA's deleterious effects. Since bacterial DNA can cause extensive immune system activation and inflammatory damage, antibodies may serve to attenuate this action by binding DNA and promoting its clearance and elimination. In this context, the IgG2 isotype, which does not fix complement well, may prevent immune-mediated damage from immune complexes which theoretically could form from chromosomal material released from dead cells.

The second question relates to the role of bacterial DNA in the pathogenesis of SLE. As suggested by the serological findings of SLE and NHS sera, autoreactivity could result from a distortion in the specificity of antibodies induced to bacterial DNA, with SLE characterized by reactivity to backbone

structures shared with self DNA. This skewing in specificity could result from aberrations in the B-cell repertoire arising from impairment in tolerance or signaling thresholds. Studies on NZB/NZW mice support this possibility since immunization with EC DNA can induce anti-dsDNA autoantibodies under conditions in which mammalian dsDNA is inactive (24).

The switch in anti-DNA specificity in SLE may relate to a mechanism other than crossreactivity. As noted previously, SLE sera lack antibodies specific for bacterial DNA. If these antibodies were important in the immune elimination of immunostimulatory material, in their absence, bacterial DNA could persist in the system and promote immune cell activation. This activation could culminate in autoreactivity as observed with other polyclonal activators such as LPS. According to this scenario, a defect in normal host defense could initiate or potentiate autoimmunity by failing to eliminate a potent foreign antigen. As bacterial DNA shares structures with self DNA, the stage would be set for induction of responses by a molecular mimicry.

The third question relates to the safety of DNA when administered as DNA vaccines or adjuvants. Specifically, can such DNA induce a pathogenic response? Although this possibility has been a matter of considerable speculation, the data reviewed herein suggests that such concerns may be unfounded. Thus, exposure to bacterial DNA containing ISS is a common, if not universal, experience of the mammalian host which, in normal individuals, leads to a noncrossreactive response. Furthermore, in normal immunity the induced antibodies display the IgG2 isotype which retards pathogenicity because of the limited ability to fix complement. Although an individual predisposed to autoimmunity may be prone to crossreactive anti-DNA responses, the risk does not appear greater than normal encounters with foreign DNA. Studies are currently in progress to define these responses further whether induced during infection, colonization or therapeutic use of DNA.

REFERENCES

1. Pisetsky, D. S. (1992) Anti-DNA antibodies in systemic lupus erythematosus. *Rheum. Dis. Clin. North Am.* **18**, 437–454.
2. Diamond, B., Katz, J. B., Paul, E., Aranow, C., Lustgarten, D., and Scharff, M. D. (1992) The role of somatic mutation in the pathogenic anti-DNA response. *Annu. Rev. Immunol.* **10**, 731–757.
3. Radic, M. Z. and Weigert, M. (1994) Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu. Rev. Immunol.* **12**, 487–520.
4. Pisetsky, D. S. (1996) Immune activation by bacterial DNA: a new genetic code. *Immunity*. **5**, 303–310.

5. Tokunaga, T., Yamamoto, T., and Yamamoto, S. (1999) How BCG led to the discovery of immunostimulatory DNA. *Jpn. J. Infect. Dis.* **52**, 1–11.
6. Pisetsky, D. S. (1998) Antibody responses to DNA in normal immunity and aberrant immunity. *Clin. Diag. Lab. Immunol.* **5**, 1–6.
7. Stollar, D., Levine, L., and Marmur, J. (1962) Antibodies to denatured deoxyribonucleic acid in lupus erythematosus serum. *Biochim. Biophys. Acta.* **61**, 7–18.
8. Sano, H. and Morimoto, C. (1982) DNA isolated from DNA/anti-DNA antibody immune complexes in systemic lupus erythematosus is rich in guanine-cytosine content. *J. Immunol.* **3**, 1341–1345.
9. Hermann, M. Winkler, T. H., Fehr, H., and Kalden, J. R. (1995) Preferential recognition of specific DNA motifs by anti-double-stranded DNA autoantibodies. *Eur. J. Immunol.* **25**, 1897–1904.
10. Karounos, D. G., Grudier, J. P., and Pisetsky, D. S. (1988) Spontaneous expression of antibodies to DNA of various species origin in sera of normal subjects and patients with systemic lupus erythematosus. *J. Immunol.* **140**, 451–455.
11. Robertson, C. R., Gilkeson, G. S., Ward, M. M., and Pisetsky, D. S. (1992) Patterns of heavy and light chain utilization in the antibody response to bacterial DNA in normal human subjects and patients with SLE. *Clin. Immunol. Immunopathol.* **62**, 25–32.
12. Robertson, C. R. and Pisetsky, D. S. (1992) Specificity analysis of antibodies to single-stranded micrococcal DNA in the sera of normal human subjects and patients with systemic lupus erythematosus. *Clin. Exp. Rheum.* **10**, 589–594.
13. Gilkeson, G. S., Grudier, J. P., Karounos, D. G., and Pisetsky, D. S. (1989) Induction of anti-double stranded DNA antibodies in normal mice by immunization with bacterial DNA. *J. Immunol.* **142**, 1482–1486.
14. Gilkeson, G. S., Grudier, J. P., and Pisetsky, D. S. (1989) The antibody response of normal mice to immunization with single-stranded DNA of various species origin. *Clin. Immunol. Immunopathol.* **51**, 362–371.
15. Halpern, M. D., Kurlander, R. J., and Pisetsky, D. S. (1996) Bacterial DNA induces murine interferon- γ production by stimulation of interleukin-12 and tumor necrosis factor- α . *Cell. Immunol.* **167**, 72–78.
16. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J., and Krieg, A. M. (1996) CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon γ . *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
17. Tighe, H., Corr, M., Roman, M., and Raz, E. (1998) Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today.* **19**, 89–99.
18. Wu, Z.-Q., Drayton, D., and Pisetsky, D. S. (1997) Specificity and immunochemical properties of antibodies to bacterial DNA in sera of normal human subjects and patients with systemic lupus erythematosus (SLE). *Clin. Exp. Immunol.* **109**, 27–31.
19. Pisetsky, D. S., and Drayton, D. M. (1997) Deficient expression of antibodies specific for bacterial DNA by patients with systemic lupus erythematosus. *Proc. Assoc. Amer. Phys.* **109**, 237–244.
20. Pisetsky, D. S., Drayton, D. M., and Wu, Z.-Q. (1999) Specificity of antibodies to bacterial DNA in the sera of normal human subjects and patients with systemic lupus erythematosus. *J. Rheum.* **26**, 1934–1983.

21. Papalian, M., Lafer, E., Wong, R., and Stollar, B. D. (1980) Reaction of systemic lupus erythematosus antinative DNA antibodies with native DNA fragments from 20 to 1,200 base pairs. *J. Clin. Invest.* **65**, 469–477.
22. Pisetsky, D. S. and Reich, C. F. (1998) The binding of anti-DNA antibodies to phosphorothioate oligonucleotides in a solid phase immunoassay. *Mol. Immunol.* **35**, 1161–1170.
23. Pisetsky, D. S. and Gonzalez, T. C. (1999) The influence of DNA size on the binding of antibodies to DNA in the sera of normal human subjects and patients with systemic lupus erythematosus. *Clin. Exp. Immunol.* **116**, 354–359.
24. Gilkeson, G. S., Pippen, A. M. M., and Pisetsky, D. S. (1995) Induction of cross-reactive anti-dsDNA antibodies in preautoimmune NZB/NZW mice by immunization with bacterial DNA. *J. Clin. Invest.* **95**, 1398–1402.

Inflammatogenic Properties of Immunostimulatory DNA Sequences

**Andrej Tarkowski, L. Vincent Collins, Guo-Min Deng,
and Lena Mölne**

“There is no excellent beauty that hath not some strangeness in the proportion” — Francis Bacon.

1. INTRODUCTION

1.1. ISS-Promoted Immunomodulation

The classical perception of DNA simply as hereditary material encoding amino acids and directing cellular functions has been dramatically challenged with the discoveries that DNA has potent immunostimulatory and inflammatogenic properties. Immunostimulatory DNA sequences (ISS) are now known to act on many different immune cell types, including B, T, dendritic, natural killer (NK) and monocytic cells (1–3). The activation of immune cells by ISS can have either beneficial (DNA vaccination, adjuvancity) or detrimental (inflammatory) effects. This dichotomy in activities reflects the multifunctionality of DNA molecules to induce both protective and destructive events.

In this review we define components of DNA structure that confer inflammatogenic properties. We review what is known about the immunomodulatory effects of ISS and describe how animal models have been used to elucidate the roles played by exogenous and endogenous DNAs in inflammatory diseases. We conclude with a discussion of the potential problems facing applications such as adjuvants, antisense, and gene therapies, where large amounts of DNA would be injected into humans, and we look at proposals as to how these difficulties might be avoided or overcome.

2. DNA AS AN ACTIVATOR OF INNATE AND ACQUIRED IMMUNITY

We define ISS as any DNA material, be it synthetic (oligonucleotides with or without modifications) or natural (DNA of bacterial, viral or eukaryotic origin), that provokes a response from immune cells either *in vivo* or *in vitro*. Synthetic oligonucleotides (ODN) have been widely used to study the *in vitro* effects on cells of defined DNA sequences. They have the additional advantages of being cheap to produce and modify and can be prepared free of contaminating compounds. ODN's containing the cytosine-phosphate-guanosine (CpG) motif have demonstrated immunostimulatory activities *in vitro* and *in vivo* (2). Modifications such as methyl, bromo or iodo residues to cytosine (4) or that incorporate nuclease-resistant backbone structures (5) have dramatic effects on the immunostimulatory properties of ODN. Phosphorothioated ODNs (S-ODNs) are significantly more resistant to degradation by nucleases compared to phosphodiester-containing DNA, and are powerful stimulators of macrophages and B and T cells *in vitro*. S-ODNs are also potent inflammatogens *in vivo* and can induce arthritis independent of the presence of any CpG motifs (Bjersing and Collins, unpublished).

Natural DNA comprises sequences from bacterial, viral, and mammalian sources. It is fascinating that mammals have evolved immune sentinel systems that detect specific types of exogenous DNA and mount protective responses. This discrimination by the host immune system between, for example, its own nuclear DNA and foreign bacterial DNA, appears to be based on two main criteria. Firstly, whereas bacterial CpG sequences are typically unmethylated at cytosine residues, eukaryotic nuclear DNA contains cytosines that are predominately methylated. Secondly, eukaryotic genomes have significantly lower frequencies of the CpG dinucleotide compared to bacterial genomes, a phenomenon known as "CpG suppression." Bacterial DNA and ODNs containing unmethylated CpG dinucleotides (CpG-ODN) induce a range of host immune responses including:

1. B-cell proliferation.
2. Cytokine secretion from B cells, T cells, NK cells, and monocytes
3. Resistance to apoptosis (6,7).

CpG-ODN's directly activate dendritic cells (8) and macrophages while activation of T cells and NK cells requires accessory signals. In the case of purified T cells, ligation of the T-cell receptor is a prerequisite for activation (7,9).

It seems likely that CpG suppression is an evolutionary tactic on the part of eukaryotic cells to avoid the induction of deleterious, anti-self immune responses. This assumption may be an over-simplification since the distinction between foreign and self-DNA is blurred in the case of mammalian mito-

chondrial DNA (mtDNA). MtDNA has similarities to bacterial DNA and differs from eukaryotic nuclear DNA (nDNA) in that it is not protected by histones, and has a higher frequency of CpG motifs that are unmethylated. When injected intraarticularly in mice, purified mtDNA induced arthritis whereas nuclear DNA from the same cells did not (Collins et al. submitted), indicating that mammalian cells contain ISS, which under certain conditions, propagate inflammatory disease.

3. IMMUNOMODULATORY DNA THERAPIES

Immunomodulation by CpG motifs has been exploited in two main ways:

1. In abrogating aberrant inflammatory responses to therapeutically injected DNA.
2. In boosting the immune response to DNA vaccines.

The Th1 promoting adjuvanticity of CpG-ODN's has also been employed with some success in the treatment of asthma in a murine model (10), and in the protection of mice against lethal *Leishmania major* infection (11,12).

The adjuvanticity of CpG-ODNs in enhancing the immunogenicity of coadministered protein antigens in mice has been well documented. In a further application, the immunogenicity of formalin-inactivated influenza virus was enhanced when administered in combination with CpG sequences (13).

The potential application of CpG-ODNs towards attenuating tumor growth is particularly exciting. CpG-ODNs have high potency in directing Th1-like responses to tumor antigens (14) and multiple doses of cationic lipids complexed with CpG-containing DNA were effective in blocking tumor growth and reestablishment (15).

4. DNA TRIGGERED IMMUNE ACTIVATION: THE DOWNSIDE

Having described some of the activities of ISS it seems reasonable to assume that in some cases, provocation of the immune system might lead to aberrant or destructive outcomes. Indeed, recent experiences with gene therapy involving intrapulmonary deposition of plasmids containing unmethylated CpGs bear this out (16,17). Therefore, reduction in the number of proinflammatory ISS in plasmids destined for gene therapy appears to be a prerequisite to avoid tissue damage (18).

Aside from the ability to induce lethal shock, there is also growing evidence that DNA sequences containing unmethylated CpG motifs can trigger inflammatory diseases such as arthritis (19), meningitis (19a), and lower airway inflammation (20) and can enhance sensitivity to LPS toxicity (21) in mice. In each of these cases of DNA-induced inflammation elevated levels of proinflammatory cytokines, such as TNF- α , have been noted. These

models are discussed in greater detail in section B. It is also noteworthy that CpG-ODNs aggravated collagen-induced arthritis in mice (22).

Although DNA from specific viral (e.g., HTLV-I) and bacterial (e.g., *Borrelia*, *Chlamydia* spp.), sources has been detected in the joints of arthritis sufferers (23,24) there is considerable debate as to whether or not these nucleic acids play direct roles in inflammation and joint destruction (25–27). Similarly, DNA has been detected in the cerebrospinal fluid (CSF) of patients suffering from meningococcal meningitis (28), and cytomegalovirus encephalitis (29), but the potential link between the presence of DNA and the inflammation has not been clarified. In the case of systemic lupus erythematosus (SLE), where patients have elevated levels of hypomethylated CpG's in the circulation, it has been suggested that microbial DNA could be a pathogenic factor (30). Although polyoma John Cunningham (JC) virus (JC) virus DNA (31) has been found in the CSF of some multiple sclerosis (MS) patients, a causal link with disease has not been established.

5. MODELS OF ISS-TRIGGERED INFLAMMATION

ISS and bacterial DNA are capable of triggering both localized and systemic inflammatory reactions. Systemic injection of ISS in mice having chemically-induced debilitation of liver function resulted in a generalized inflammatory response leading to septic shock (32). This state was clearly mediated by the release of TNF- α , a cytokine known to mediate symptoms of septic shock consequent to both Gram-positive and -negative bacteremias.

Moreover, in mice with intact liver function, ISS present in bacterial genomes (*Staphylococcus aureus* and *Escherichia coli*), or ODNs containing unmethylated CpG motifs gave inflammatory responses in various target organs. As little as 0.6 μ g of ISS, deposited intraarticularly, gave rise to arthritis in approx 90% of mice from different genetic backgrounds. Higher doses (6 μ g/joint) led to joint inflammation in all of the injected mice (19). This higher level of injected DNA represents the total nucleic acid from approx 10^6 bacteria, a quantity of microorganisms that may be easily found in joints during septic arthritis. Inflammatory cell influx was visible within hours of ISS injection, peaked within a few days and lasted for several weeks. The inflammatory infiltrate was characterized by the presence of macrophages and the absence of lymphocytes and polymorphonuclear granulocytes. Results obtained from the above studies suggest that the killing of bacteria during septic arthritis treatment might not be enough to control disease progression. Irrigation of joints to remove proinflammatory bacterial products (including ISS), combined with antiinflammatory drugs, should be valuable adjuncts in treating this debilitating disease.

Eukaryotic cells contain low levels of unmethylated CpG motifs. However, the mitochondrial genome, which is present in multiple copies, contains DNA with unmethylated cytosines. It should be stressed that mitochondria are considered to have evolved from free-living oxidative prokaryotes. Owing to the similarities regarding physiochemical properties between bacterial and mitochondrial DNA, we assessed the potential inflammatogenicity of mtDNA. Highly purified murine and human mtDNA induced arthritis in the vast majority of mice, upon a single intraarticular injection (Collins et al., submitted). In addition, PCR-amplified fragments of the mitochondrial genome displayed similar properties. In contrast, human or murine nuclear DNA lacked arthritogenic properties when injected intraarticularly. Interestingly, extracellular mitochondrial DNA can be found in the majority of inflamed joints of patients with rheumatoid arthritis (Hajizadeh et al., submitted), raising the possibility that this endogenous cell constituent might be responsible for the perpetuation of inflammation and consequently, disease chronicity. Another property of mtDNA, of great interest with respect to its inflammatogenic properties, is the presence of oxidatively damaged adducts in the genomic sequences. This is due to the proximity of mtDNA to the respiratory chain in mitochondria, the lack of protective histones, and a less efficient repair system compared to that in the nuclear compartment. *In vivo* experiments showed that oxidized DNA exerted strong and long lasting inflammatogenic properties, upon injection into knee joints (Collins et al., submitted).

Diarthrodial joints are not by any means the only targets for inflammatogenic ISS. Indeed, mucosal surfaces, such as those in lungs, may be target tissues for both pro- and anti-inflammatory ISS. Thus, intratracheal instillation of bacterial DNA, or synthetic ISS containing CpG motifs, will lead within hours, to inflammation in the lower respiratory tract. This inflammation is characterized by an influx of predominantly polymorphonuclear granulocytes, and localized production of certain proinflammatory cytokines (20). In contrast, instillation of synthetic DNA lacking CpG motifs or having methylated CpG motifs did not trigger inflammation. The possible relevance of these data to human disease was indicated in a study of cystic fibrosis patients, in which 0.1–1% of sputum DNA was of bacterial origin (20). Interestingly, systemic, rather than local administration of CpG-ODNs, led to downmodulation of an experimental model of inflammatory asthma (10). These seemingly contradictory results might reflect the different routes of ISS administration (locally vs systemically), and the potential of ISS to trigger Th1 type immune reactivity, which would be beneficial in asthma by downregulating endogenous, Th2 polarized responses.

Some other recently published studies show that the central nervous system (CNS) and skin are also target organs for ISS inflammation. Indeed, low amounts of staphylococcal DNA or synthetic ISS will cause meningitis upon intraventricular injection of these compounds into rat or mouse brains. Inflammatory responses appear within hours and last for weeks. Meningitis triggered by ISS is characterized by meningeal influx of monocytic cells. In addition, cerebrospinal fluid of rats with ISS triggered meningitis shows signs of pleocytosis and blood-brain barrier dysfunction (19a). Human skin is constantly colonized by bacteria, without any visible signs of inflammation. However, as soon as the skin barrier function is compromised, bacteria enter the dermal layers and induce a vivid inflammatory response. In this context, intradermal injection of ISS leads to a localized, macrophage mediated inflammatory response (Mölne et al., unpublished).

6. WHAT ARE THE ISS EFFECTOR MECHANISMS?

The mechanisms underlying ISS-triggered inflammation have been dissected in great detail. In the case of ISS-triggered lower respiratory tract inflammation, pathology is apparently mediated by neutrophil granulocytes, although no deletion experiments were performed (20). In contrast, ISS-mediated arthritis and meningitis are easily induced in the absence of neutrophil granulocytes and lymphocytes i.e., B, T and NK cells (19,19a). However, deletion of circulating monocytes, using etoposide, abrogates inflammation in the joints and in the brain (19,19a). ISS-provoked, monocyte-mediated arthritis is triggered by activation of transcription factor NF κ B, as evidenced by experiments in mice showing that antisense ODNs to the NF κ B p65 subunit downregulated joint inflammation (33). Corroborating this finding, knockout mouse defective for TNF- α , an important proinflammatory cytokine that is controlled by NF κ B, did not develop arthritis following intraarticular ISS injection (19).

Additional soluble mediators of inflammation are of importance in these conditions. The inhibition of NO synthesis, using the inhibitors of nitric oxide synthase L-NMMA and L-NAME, markedly downregulated ISS-triggered meningitis (19a). In contrast, short-time depletion of the complement system using cobra venom factor or interaction with monocyte-expressed complement receptor 1 did not affect the incidence or severity of meningitis.

Selectins are involved in leukocyte rolling and thus control early steps of leukocyte extravasation. We investigated whether selectins had a role to play in ISS-mediated arthritis using fucoidin, which has the ability to block P- and L-selectins. Histopathological results 24 h after inoculation of ISS demonstrated that the severity and incidence of meningitis were markedly reduced

by fucoidan treatment (19a), indicating that monocyte extravasation is mediated, at least in part, by this set of molecules.

In the case of bacterial infections, ISS release is concomitant with bacterial cell degradation and release of other inflammatogenic bacterial compounds, including peptidoglycans from Gram positive and LPS from Gram negative bacteria. We have assessed if these compounds are able, in concert with ISS, to aggravate inflammation. In this respect, suboptimal amounts of CpG ODN and either peptidoglycans or LPS were injected intraventricularly. It was apparent that these bacterial substances acted synergistically with ISS in triggering meningitis (19a).

7. PROINFLAMMATORY AND ADJUVANT PROPERTIES OF ISS DNA: SAME OR DIFFERENT EFFECTOR MECHANISMS?

As dealt with in other chapters of this book, ISS have been successfully used as adjuvants to stimulate specific immunity to protein antigens. Indeed, ISS activate dendritic cells/macrophages and are thereby instrumental in antigen presentation. However, ISS are able to trigger the activities not only of monocytic/dendritic cells, but also those of B lymphocytes. Indeed, it has been shown that cultures of B-lymphocytes even in the absence of accessory cells (34). These data suggest that ISS adjuvanticity and inflammatogenicity might operate via distinctly different mechanisms, since ISS-triggered inflammation is readily obtained in B-cell deficient SCID mice (19,19a)

8. COUNTERACTING UNDESIRABLE EFFECTS OF ISS

The best way to prevent undesired ISS effects is to avoid the administration of these molecules in places where they might be expected to exert undesired deleterious activities. This may readily be achieved with respect to joints and the CNS. However, in the case of skin, the situation is more complicated since ISS DNA will probably be coadministered together with a relevant protein/peptide immunogen. A problem is also encountered when DNA is administered systemically, where activation of inflammatory cells in the lungs can have potentially catastrophic consequences (16,17).

There are a number of potential ways to avoid ISS-induced inflammatory side-effects. Potential strategies to downregulate undesired ISS-induced inflammatogenicity involve:

1. Removal or methylation of plasmid DNA CpG motifs (18).
2. The concomitant use of mammalian DNA and/or the incorporation of suppressor sequences, such as poly-guanosine (35).

An inherent problem with these approaches is the possible downregulation, not only of inflammation but also of ISS adjuvanticity.

Since ISS exert predominantly Th1 effects (36) it might be of interest to deviate the responsiveness to Th2, thereby downregulating any undesired inflammation, while at the same time upregulating B-cell responses. This can be achieved by simultaneous administration of IL-10 and/or IL-4. Such an approach might not counteract the ISS adjuvanticity, at least not with respect to antigen-specific antibody responses.

9. PERSPECTIVES

The future of DNA-based therapies, vaccinations, and immunomodulation looks promising but will be hindered, at least in humans, by serious concerns regarding aberrant and undesired immunodeviation. Based on our current knowledge of ISS adjuvanticity and inflammatogenicity it seems wise to assume that there may be drawbacks to DNA therapies. It is increasingly clear that exogenous DNA provokes immune reactions that can be amplified into tissue-destructive events in various organs. The significance for human disease of ISS-driven responses is still unclear, but it is likely that certain ISS combinations are instigators or exacerbators of inflammation in specific organs. Careful scrutiny needs to be made of the *in vivo* immunostimulatory properties of DNA derivatives having modifications, that enhance *in vivo* persistence, e.g., phosphorothioate, methylphosphonate, and 2'-O-methylribonucleotide substitutions (5,37), and bioavailability (e.g., aptamers: peptide-DNA chimerae). Aptamers have greater stability and enhanced binding compared to ODN's. Recent findings that aptamers can breach the blood brain barrier (38), raises both the exciting possibility of new gene therapies and the scepter of adverse inflammatory events in the brain.

Similarly, we need to look more closely at our own, mammalian, DNA to uncover the secrets of endogenous proinflammatory mtDNA and to define the counteracting, antiinflammatory sequences on nuclear DNA. Endogenous ISS released in pathological situations of cell necrosis and apoptosis might well be important factors in inflammatory diseases of humans. Indeed, auto-regulation of endogenous DNA immunostimulation may represent a mechanism of immune priming of self by maintaining a basal level of inflammation, and disruption of this equilibrium might lead to aberrant immune function.

REFERENCES

1. Heeg, K. and Zimmermann, S. (2000) CpG DNA as a Th1 trigger. *Int. Arch. Allergy Immunol.* **121**, 87–97.
2. Scheule, R. K. (2000) The role of CpG motifs in immunostimulation and gene therapy. *Adv. Drug Deliv. Rev.* **44**, 119–134.

3. Stacey, K. J., Sester, D. P., Sweet, M. J. and Hume, D.A. (2000) Macrophage activation by immunostimulatory DNA. *Curr. Top Microbiol. Immunol.* **247**, 41–58.
4. Boggs, R. T., McGraw, K., Condon, T., et al. (1997) Characterization and modulation of immune stimulation by modified oligonucleotides. *Antisense Nucleic Acid Drug Dev.* **7**, 461–471.
5. Krieg, A. M., Matson, S., and Fisher, E. (1996) Oligodeoxynucleotide modifications determine the magnitude of B cell stimulation by CpG motifs. *Antisense Nucleic Acid Drug Dev.* **6**, 133–139.
6. Krieg, A. M., Yi, A. K., Schorr, J., and Davis, H. L. (1998) The role of CpG dinucleotides in DNA vaccines. *Trends Microbiol.* **6**, 23–27.
7. Lipford, G. B., Heeg, K., and Wagner, H. (1998) Bacterial DNA as immune cell activator. *Trends Microbiol.* **6**, 496–500.
8. Sparwasser, T., Koch, E. S., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
9. Lipford, G. B., Bauer, M., Blank, C., Reiter, R., Wagner, H., and Heeg, K. (1997) CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344.
10. Kline, J. N., Waldschmidt, T. J., Businga, T. R., et al. (1998) Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* **160**, 2555–2559.
11. Lipford, G. B., Sparwasser, T., Bauer, M., et al. (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* **27**, 3420–3426.
12. Zimmermann, S., Egeter, O., Hausmann, S., et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* **160**, 3627–3630.
13. Cohen, A. D., Boyer, J. D. and Weiner, D. B. (1998) Modulating the immune response to genetic immunization. *FASEB J.* **12**, 1611–1626.
14. Kim, S. K., Ragupathi, G., Musselli, C., Choi, S. J., Park, Y. S. and Livingston, P. O. (1999) Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. *Vaccine* **18**, 597–603.
15. Lanuti, M., Rudginsky, S., Force, S. D., et al. (2000) Cationic lipid:bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models. *Cancer Res.* **60**, 2955–2963.
16. Yew, N. S., Wang, K. X., Przybylska, M., et al. (1999) Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid:pDNA complexes. *Hum. Gene Ther.* **10**, 223–234.
17. Li, S., Wu, S. P., Whitmore, M., et al. (1999) Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am. J. Physiol.* **276**, L796–804

18. Yew, N. S., Zhao, H., Wu, I. H., et al. (2000) Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol. Ther.* **1**, 255–262.
19. Deng, G.-M., Nilsson, I. M., Verdreng, M., Collins, L. V. and Tarkowski, A. (1999) Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nature Med.* **5**, 702–705.
- 19a. Deng, G.-M., Liu Z.-Q., Tarkowski, A. (2001) Intracisternally localized bacterial DNA containing CpG motifs induces meningitis. *J. Immunol.* **167**, 4616–4626.
20. Schwartz, D. A., Quinn, T. J., Thorne, P. S., Sayeed, S., Yi, A. K. and Krieg, A. M. (1997) CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* **100**, 68–73.
21. Cowdery, J. S., Chace, J. H., Yi, A. K. and Krieg, A. M. (1996) Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. *J. Immunol.* **156**, 4570–4575.
22. Miyata, M., Kobayashi, H., Sasajima, T., Sato, Y. and Kasukawa, R. (2000). Unmethylated oligo-DNA containing CpG motifs aggravates collagen-induced arthritis in mice. *Arthritis Rheum.* **43**, 2578–2582.
23. Bas, S., Griffais, R., Kvien, T. K., Glennas, A., Melby, K. and Vischer, T. L. (1995) Amplification of plasmid and chromosome Chlamydia DNA in synovial fluid of patients with reactive arthritis and undifferentiated seronegative oligoarthritis. *Arthritis Rheum.* **38**, 1005–1013.
24. Nocton, J. J., Dressler, F., Rutledge, B. J., Rys, P. N., Persing, D. H. and Steere, A. C. (1994) Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *N. Engl. J. Med.* **330**, 229–234.
25. Kingsley, G. (1997) Microbial DNA in the synovium: a role in aetiology or a mere bystander? *Lancet* **349**, 1038–1039.
26. Soderlund, M., von Essen, R., Haapasaari, J., Kiistala, U., Kiviluoto, O. and Hedman, K. (1997) Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. *Lancet* **349**, 1063–1065.
27. Wuorela, M. and Granfors, K. (1998) Infectious agents as triggers of reactive arthritis. *Am. J. Med. Sci.* **316**, 264–270.
28. Kotilainen, P., Jalava, J., Meurman, O., et al. (1998) Diagnosis of meningococcal meningitis by broad-range bacterial PCR with cerebrospinal fluid. *J. Clin. Microbiol.* **36**, 2205–2209.
29. Prosch, S., Schielke, E., Reip, A., et al. (1998) Human cytomegalovirus (HCMV) encephalitis in an immunocompetent young person and diagnostic reliability of HCMV DNA PCR using cerebrospinal fluid of nonimmunosuppressed patients. *J. Clin. Microbiol.* **36**, 3636–3640.
30. Krieg, A. M. (1995) CpG DNA: a pathogenic factor in systemic lupus erythematosus? *J. Clin. Immunol.* **15**, 284–922.
31. Ferrante, P., Omodeo-Zorini, E., Caldarelli-Stefano, R., et al. (1998) Detection of JC virus DNA in cerebrospinal fluid from multiple sclerosis patients. *Mult. Scler.* **4**, 49–54.

32. Sparwasser, T., Miethke, T., Lipford, G., et al. (1997) Bacterial DNA causes septic shock. *Nature* **386**, 336–337.
33. Deng, G-M., Verdrengh, M., Liu, Z-Q. and Tarkowski, A. (2000) The major role of macrophages and their product TNF- α in induction of arthritis triggered by CpG motifs in bacterial DNA. *Arthritis Rheum.* **43**, 2283–2289.
34. Krieg, A. M., Yi, A-K., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549.
35. Pisetsky, D. S. and Reich C. F. (2000) Inhibition of murine macrophage IL-12 production by natural and synthetic DNA. *Clin. Immunol.* **96**, 198–204.
36. G. Krieg, A. M. and Wagner, H. (2000) Causing a commotion in the blood: immunotherapy progresses from bacteria to bacterial DNA. *Immunology Today* **21**, 521–526.
37. Zhao, Q., Temsamani, J., Iadarola, P. L., Jiang, Z. and Agrawal, S. (1996) Effect of different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem. Pharmacol.* **51**, 173–182.
38. Tyler, B. M., Jansen, K., McCormick, D. J., et al. (1999) Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood-brain barrier and specifically reduce gene expression. *Proc. Natl. Acad. Sci. USA* **96**, 7053–7058.

The Role of Immunostimulatory DNA Sequences in Arthritis

**Sarah T.A. Roord, Arash Ronaghy,
Berent J. Prakken, Kenji Takabayashi,
and Dennis A. Carson**

1. INTRODUCTION

Joint destruction in rheumatoid arthritis is caused by an invasive pannus, composed of activated macrophages and fibroblasts. The disease is thought to have an immunological origin because of

1. The abundant immune complexes, complement split products, and rheumatoid factor autoantibodies in affected joints,
2. The close association of the disease with HLA DR β 1 types that express the QKRAA shared epitope,
3. The accumulation within the joints of activated lymphocytes and monocytes.

Despite intensive investigations over more than 30 yr, no exogenous or endogenous antigen that drives rheumatoid synovitis has been identified. Rheumatoid arthritis looks like an infectious disease with an autoimmune component, but no specific infectious organisms have been isolated from the joints. These results raise the possibility that rheumatoid arthritis may be triggered by transient exposure to one or more bacteria or viruses via a “hit-and-run” mechanism, and then perpetuated because of an abnormal host response, which becomes self-sustaining. Hence, a major aim of rheumatoid arthritis research is to determine how foreign antigens can interact with a predisposed host to induce an abnormal synovial environment conducive to persistent inflammation.

In addition to providing a source of exogenous antigens, that may crossreact with those of the host, bacteria can exert an adjuvant effect on immune responses. Classic examples are the bacterial lipopolysaccharides,

that induce monocytes to release IL-1 and TNF α , and the bacterial "superantigens" that trigger T- and B-lymphocyte mitogenesis. Although such adjuvant effects certainly could be sufficient to start a systemic inflammatory response, their effects would not be expected to be permanent. The central problem remains: Why should a host's inflammatory response persist after withdrawal of the initiating exogenous stimulus?

There are two critical features of ISS (Immunostimulatory DNA Sequences) that differentiate them from bacterial superantigens and lipopolysaccharides: They generally are not immunogenic, and they interact with intracellular targets. DNA can persist within cells in an unintegrated state for a prolonged period, hidden from the immune system (1). Consequently, the *in vivo* Th1 imprinting effects of ISS can potentially last a long time, compared to the effects of other adjuvants. Once the Th1 to Th2 memory T-cell ratio reached high levels, boosting with protein antigen could no longer deviate the immune response toward Th2. Through such a hit and run mechanism, bacterial ISS could initiate a stable Th1 delayed hypersensitivity response to common antigens on normally harmless skin and gut bacteria, in the absence of a chronic infection.

2. DOES ISS PLAY A ROLE IN ARTHRITIS?

There is as of yet, no proof that bacterially derived ISS play a role in rheumatoid arthritis. However, circumstantial data are consistent with this possibility. As summarized earlier, the two most consistent immune abnormalities in the affected joints of rheumatoid arthritis patients are high affinity rheumatoid factor autoantibodies, and T lymphocytes reactive with bacterial heat shock proteins. Rheumatoid factors are serological markers of previous immune complex formation, and typically occur during bacterial or parasitic infections. Heat shock proteins are dominant bacterial antigens, and many also play a role in the transfer of antigens to specialized antigen presenting cells. Moreover, the *dnaJ* class of bacterial heat shock proteins contain the QKRAA shared epitope that predisposes to rheumatoid arthritis (17). The skin and mucous membranes supply the immune system with a continual source of bacteria containing both ISS and *dnaJ*. Normally the immune response to gut bacteria and other mucosal antigens is polarized toward a Th2 phenotype. In contrast, data from Albani and colleagues indicate that T cells from rheumatoid arthritis patients, that are reactive with *dnaJ*, display a Th1 phenotype.

The relevance of acute experimental models of arthritis and autoimmunity to naturally occurring human diseases is questionable. However, all antigen-induced forms of arthritis in rodents require the coadministration of

a macrophage activating adjuvant (typically complete Freund's adjuvant [CFA]). ISS have been reported to mimic the effects of CFA, and indeed can exacerbate autoimmunity in several models (2,3).

Sensitive PCR studies have failed to reveal any evidence for bacterial ribosomal sequences in the joints of rheumatoid arthritis patients. Most of the DNA in inflammatory synovial fluids probably derives from dead neutrophils and mononuclear cells. Early work showed that DNA released by dying cells induced cytokine production and lymphocyte proliferation. Sato and colleagues recently have reported that DNA isolated from the synovial fluids of rheumatoid patients displayed strong monocyte activating activity (4). Such sequences could derive from released mitochondrial DNA, that has unmethylated CpG motifs, or from oxidatively modified DNA released by dead neutrophils. Several years ago, Mond and colleagues reported that 8-oxoguanosine had strong immunostimulatory activity toward mouse B cells (5). Subsequent synthetic studies of Robins, Cottam and coworkers with a variety of purine analogs confirmed the ISS activity of 8-oxopurines and deazapurines (6), while Goodman and coworkers showed that modified guanosine induced synthesis of the p40 chain of IL-12 (7). At the time of these studies, the division of immune responses into Th1 and Th2 types was not appreciated, and the potential effects of oxidation on the ISS activity of mammalian DNA and RNA were not directly studied. However, recent investigation have revealed that superoxide, hydrogen peroxide, hypochlorite, and nitric oxide, that are produced by activated phagocytes, induce numerous modifications of guanine and cytosine residues in DNA (8,9).

Collectively, these studies provide a rationale for not only the initiation of rheumatoid synovitis, but for the maintenance of the disease. According to this hypothesis, genetically susceptible individuals who express the shared epitope are prone to develop a strong immune response to the *dnaJ* heat shock proteins expressed by bacteria on skin and mucus membranes. Although this response normally is skewed toward a Th2 phenotype, if the immune system is exposed to antigen in the presence of bacterial ISS, macrophages and antigen presenting cells release IL-12, that causes antigen specific Th0 precursors to differentiate to Th1 memory and effector cells. At disease onset, noninfectious fragments of bacterial ISS might travel with monocyte precursors of synovial lining cells into the joints, initiating localized delayed hypersensitivity reactions. Subsequently, oxygen radicals released by dead neutrophils within the inflammatory, confined, synovial space would facilitate the formation of ISS from human DNA. Even if the oxidized DNA had less potent activity than bacterial ISS, they might raise the ISS to immunoinhibitory sequences ratio, to a critical threshold sufficient to maintain the synovial pannus.

Recently, Tarkowski and coworkers were able to link joint damage in a model of septic arthritis to the presence of ISS (10). They showed that direct injection of bacterial DNA or oligonucleotides containing ISS into the joints led to arthritis, whereas injection of mammalian DNA did not induce inflammation. An influx of monocytes and macrophages, and only a minority of CD4 positive T lymphocytes, characterized the resulting arthritis. Locally, in the arthritic joint, an increased expression was found of mRNA for TNF- α , IL-1 α and IL-12 and for the chemokines RANTES and MCP-1 (11). Altogether, these results indicated that bacterial DNA was sufficient to provoke joint inflammation in septic arthritis, and raised the question whether ISS might also play a role in the pathogenesis of other forms of arthritis (11).

To explore this issue, we turned to a T-cell mediated model of experimental autoimmune arthritis, namely adjuvant arthritis (AA) (12). Adjuvant arthritis is an extensively studied form of chronic arthritis with a close histopathological resemblance to rheumatoid arthritis (13,14). AA can be induced in susceptible animals such as Lewis rats by injection of heat-killed Mtb in incomplete Freund's adjuvant (IFA) in the base of the tail. Since the immunostimulatory properties of bacterial DNA were first discovered in Mtb (15), we reasoned that ISS might play a role in this model of autoimmune arthritis.

3. RESULTS

First, we determined whether the presence of Mtb DNA is necessary to induce adjuvant arthritis. We treated heat-killed Mtb with DNase until no high mw DNA was detectable by electrophoresis and ethidium bromide staining. As shown in Fig. 1, we injected Lewis rats with either emulsified heat killed Mtb, DNase treated Mtb, DNase treated Mtb supplemented with ISS-ODN (3, 10, 30, and 100 μ g), DNase treated Mtb with a control ODN, or IFA mixed with ISS. The results are shown in Table 1. DNase treatment of Mtb lead to a delay in the onset and a marked reduction in the severity of arthritis (mean maximum arthritis score 5) compared to the positive control (mean maximum arthritis score 12, $p < 0.0001$). The addition of ISS-ODN (100 μ g) to DNase treated Mtb restored the severity of arthritis completely (mean maximum arthritis score 12), whereas, the addition of control ODN was devoid of efficacy ($p < 0.0001$). The clinical findings were also reflected in the histological scores (Table 2). These experiments showed that the severity of joint inflammation in AA depends on the presence of Mtb DNA.

Systemic immunization with ISS, mixed with IFA alone, was not sufficient to induce arthritis in Lewis rats. Rats immunized with ISS and IFA

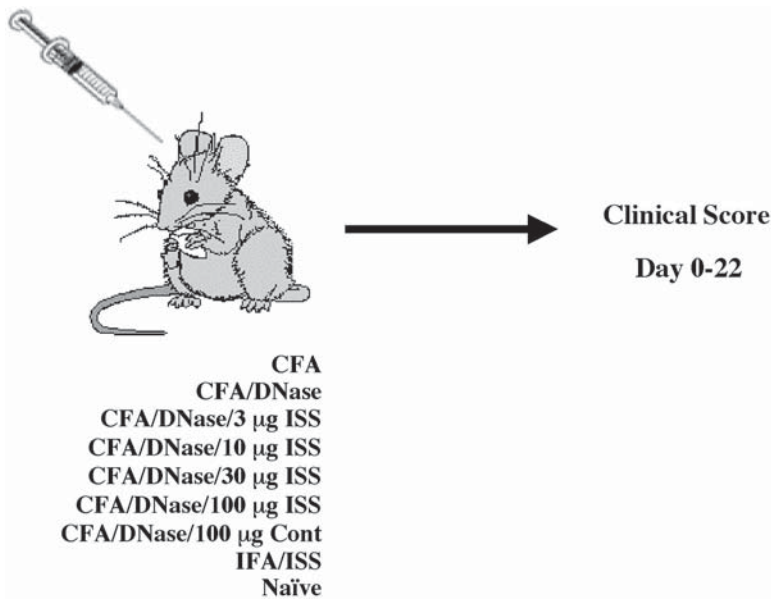


Fig. 1. Experimental set-up Adjuvant Arthritis. Lewis rats were injected in the base of the tail with either emulsified heat killed Mtb, DNase treated Mtb, DNase treated Mtb supplemented with ISS-ODN (3, 10, 30, and 100 µg), DNase treated Mtb with a control ODN or IFA mixed with ISS-ODN. Clinical arthritis scores were assessed on d 0–22.

Table 1
ISS Present in CFA Determine the Clinical severity of Adjuvant Arthritis

Groups	Day 2	Day 10	Day 14	Day 18	Day 22
CFA	0 ± 0	2.1 ± 0.69	8.4 ± 1.5	11.3 ± 1.69	11.8 ± 1.75
CFA/DNase	0 ± 0	0 ± 0	2.5 ± 0.5	4.5 ± 1.36	5.4 ± 1.43#
CFA/DNase+ 3 µg ISS-ODN	0 ± 0	0.4 ± 0.1	3.5 ± 0.67	7.2 ± 1.21	8.5 ± 1.54
CFA/DNase+ 10 µg ISS-ODN	0 ± 0	0.2 ± 0.12	5.5 ± 0.45	8.5 ± 0.87	10.3 ± 0.34
CFA/DNase+ 30 µg ISS-ODN	0 ± 0	0.3 ± 0.12	8.1 ± 0.64	11.4 ± 1.17	11.2 ± 1.02
CFA/DNase+ 100 µg ISS-ODN	0 ± 0	2.5 ± 0.63	9 ± 1.23	10.9 ± 1.34	12.3 ± 1.33
CFA/DNase+ 100 µg Cont-ODN	0 ± 0	0 ± 0	0 ± 0	0.83 ± 0.46	1.7 ± 0.6#
IFA/ISS	0 ± 0	0 ± 0	0 ± 0	0.25 ± 0.14	0.63 ± 0.38
Naïve	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Lewis rats were immunized with CFA, CFA containing DNase treated Mtb, CFA containing DNase treated Mtb mixed with 3 µg, 10 µg, 30 µg or 100 µg of ISS-ODN, CFA containing DNase treated Mtb mixed with 100 µg of control ODN or IFA mixed with ISS. Shown are the mean arthritis scores (± SEM) at the indicated times after the immunization with the above mentioned antigens (12).

$p < 0.0001$ vs CFA

Table 2
ISS Determine the Pathologic Severity of Adjuvant Arthritis

Groups	Mean histology score
CFA	10.5 ± 1.26
CFA/DNAse	5 ± 0.71
CFA/DNAse+100 µg ISS-ODN	9 ± 0.71
CFA/DNAse+100 µg Cont-ODN	3.75 ± 1.88
IFA/ISS	2 ± 0.58
Naive	1 ± 0

Lewis rats were immunized with CFA, CFA containing DNAse treated Mtb, CFA containing DNAse treated Mtb mixed with 100 µg of ISS-ODN, CFA containing DNAse treated Mtb mixed with control ODN or IFA mixed with ISS. On d 22 after the injection with these antigens, rats were sacrificed and their ankles were decalcified and paraffin-embedded. Shown are the mean histological scores ± SEM (12).

showed normal weight curves (not shown), and did not display signs of arthritis up till 60 d after immunization. These results suggest that mycobacterial antigens, or other factors besides DNA, also are required for arthritis induction.

To determine the effects of Mtb DNA on antigen specific cytokine synthesis, we cultured inguinal lymph node cells (ILN) at day 55 after arthritis induction with purified Mtb hsp65. After 72 h culture, supernatants were collected and IFN-γ and RANKL levels were assayed. Results are shown in Table 3. Lymph node cells from rats immunized with whole heat killed Mtb produced IFN-γ and soluble RANKL after in vitro activation with hsp65. In contrast, lymph node cells from rats immunized with DNAse treated Mtb produced significantly less IFN-γ and RANKL after in vitro restimulation with hsp65. However, lymph node cells from rats immunized with DNAse treated Mtb, which had been supplemented with ISS-ODN, produced high levels of IFN-γ and RANKL after in vitro activation with hsp65, whereas lymph node cells from rats immunized with DNAse treated Mtb, mixed with a control ODN, produced IFN-γ and RANKL at levels comparable to those produced by cells from rats immunized with DNAse treated Mtb (DNAse-CFA/ISS-ODN vs DNAse-CFA/Cont-ODN). We could not detect antigen specific IFN-γ and RANKL production in naive rats. Other experiments demonstrated no significant production of IL-4 or IL-10 in any of the lymph node cultures. Thus, primed lymph node cells from rats immunized with a mixture containing ISS (either Mtb DNA or ISS-ODN) produced significantly higher levels of IFN-g and RANKL, compared to primed lymph node cells from rats immunized with Mtb without ISS. The levels of IFN-γ and

Table 3
Bacterial DNA Stimulates the Production of IFN- γ and RANKL in Response to Mycobacterial hsp65

Groups	IFN- γ (pg/mL)	RANKL (pg/mL)
CFA	5.5 $\pm$ 2.7	214 $\pm$ 48.9
CFA/DNAse	1.9 $\pm$ 0.3	88 $\pm$ 11.5
CFA/DNAse+100 μ g ISS-ODN	6.5 $\pm$ 1.2	169 $\pm$ 18.5
CFA/DNAse+100 μ g Cont-ODN	0.84 $\pm$ 0.1	31 $\pm$ 17.1
IFA/ISS	0 $\pm$ 0	42 $\pm$ 7.4
Naive	0.9 $\pm$ 0	16 $\pm$ 9.8

Inguinal lymph node cells (ILN) were harvested on d 55 after arthritis induction and restimulated in vitro with purified Mtb hsp65 for 72 h. Supernatants were harvested and IFN- γ and RANKL levels were assayed by ELISA. Results are the means $\pm$ SEM for four rats per group (12).

RANKL production by antigen restimulated lymph node cells correlated with the severity of arthritis found in the different treatment groups.

Because intraarticular injections of ISS induce joint inflammation, it was important to determine if Mtb DNA was present in the joints. At 1, 3, 7, 10, 14, 17, 21, 29, and 36 d after injection of Mtb into the tails of Lewis rats, PCR analyses were performed on tissue samples taken from kidney, liver, spleen, bone marrow, base of the tail, inguinal lymph node and synovium. Mycobacterial DNA was detected at the site of injection, in the spleen up until d 36, in the draining (inguinal) lymph nodes at d 10 and in the bone marrow at days 3, 7, 10, and 14. No Mtb DNA was detected in the liver and kidney, or synovium. Thus, after immunization with CFA, mycobacterial DNA disperses to bone marrow and lymphoid tissues, but not to the synovium. Hence, the arthritogenic effects of Mtb cannot be attributed to a local effect on the synovium, as was the case in the model of septic arthritis (10).

We then explored the presence of ISS-ODN in the affected synovial tissues (ankles). Rats were injected with CFA/DNase/ISS-ODN (100 μ g) and killed at d 1 and d 14 (two rats per time-point). DNA was extracted from the injected area (base of the tail) and from the inflamed ankles, and was subjected to Southern blot analysis using ³²P labeled complementary ODN as a probe. ISS-ODN was detected in the area of injection, but not in the affected synovium.

4. CONCLUSION

The results of these experiments have shown that

1. The Th1 response to mycobacterial antigens correlates with the severity of adjuvant arthritis.

2. The combination of ISS and IFA does not induce adjuvant arthritis.
3. The severity of arthritis depends on the presence of ISS.
4. In this system, arthritis development is not explained by intraarticular mycobacterial DNA.
5. ISS stimulates production of RANKL.

Thus, these experiments have revealed a new mechanism through which ISS DNA could play a role in inflammatory arthritis. The ISS induces the production of RANKL, which can stimulate breakdown of bone by osteoclasts. This may allow the entry of primitive mesenchymal cells in the bone marrow to penetrate the synovial cavity. Inhibitors of RANKL (e.g., OPG) may be able to block this process.

During the past year, the Toll like receptor 9 (TLR9) was shown to play a central role in initiating cell activation by ISS (16). Ongoing experiments in our laboratory are testing the role of TLR9, by breeding TLR9 knockout mice into arthritis-prone strains, and testing their susceptibility to joint disease. It is conceivable that TLR9 could be a target for intervention in rheumatoid arthritis, and other forms of inflammatory arthritis.

ACKNOWLEDGMENTS

This work was supported in part by grants AR44850, AR07567 and AI40682 from the National Institutes of Health. S. Roord and B. Prakken are supported by the "Ter Meulen Fund" from the Royal Netherlands Academy for Arts and Sciences. We thank Dr. Eyal Raz for his helpful comments.

REFERENCES

1. Raz, E., Carson, D. A., Parker, S. E., et al. (1994) Intradermal gene immunization: The possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* **91**, 9519–9523.
2. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. and Harding, C. V. (1997) CpG oligonucleotides act as adjuvants that switch on Th1 immunity. *J. Exp. Med.* **186**, 1623–1631.
3. Segal, B. M., Klinman, D. M. and Shevach, E. M. (1997) Microbial products induced autoimmune disease by an IL-12 dependent pathway. *J. Immunol.* **158**, 5087–5090.
4. Sato, Y., Sato, H., Saito, A., et al. (1997) CpG motifs in DNA in synovial fluids (SF) from rheumatoid arthritis patients are hypomethylated and enhance TNF- α production by SF monocytes. *Arthritis Rheum.* **40**, S78. Abstract.
5. Ahmad, A. and Mond, J. J. (1985) 8-hydroxyguanosine and 8-methoxyguanosine possess immunostimulating activity for B lymphocytes. *Cell. Immunol.* **94**, 276–280.
6. Bonnet, P. A. and Robins, R. K. (1993) Modulation of leukocyte genetic expression by novel purine nucleoside analogues. A new approach to antitumor and antiviral agents. *J. Med. Chem.* **36**, 635–653.

7. Pope, B. L., MacIntyre, J. P., Kimball, E., et al. (1995) The immunostimulatory compound 7-allyl-8-oxoguanosine (loxoribine) induces a distinct subset of murine cytokines. *Cell. Immunol.* **162**, 333–339.
8. Spencer, J. P., Wong, J., Jenner, A., Aruoma, O. I., Cross, C. E. and Halliwell, B. (1996) Base modification and strand breakage in isolated calf thymus DNA and in DNA from human skin epidermal keratinocytes exposed to peroxynitrite or 3-morpholinocydonimine. *Chem. Res. Toxicol.* **9**, 1152–1158.
9. Whiteman, M., Jenner, A. and Halliwell, B. (1997) Hypochlorous acid-induced base modifications in isolated calf thymus DNA. *Chem. Res. Toxicol.* **10**, 1240–1246.
10. Deng, G. M., Nilsson, I. M., Verdrengh, M., L. Collins, V. and Tarkowski, A. (1999) Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nat. Med.* **5**, 702.
11. Deng, G. M. and Tarkowski, A. (2000) The features of arthritis induced by CpG motifs in bacterial DNA. *Arthritis Rheum.* **43**, 356.
12. Ronaghy, A., Prakken, B. J., Takabayashi, K., et al. (2002) Immunostimulatory DNA sequences influence the course of adjuvant arthritis. *J. Immunol.* **168**, 51–56.
13. Pearson, C. M. (1956) Development of arthritis, peri-arthritis and periostitis in rats giving adjuvant. *Proc. Soc. Exp. Biol. Med.* **91**, 91.
14. Pearson, C. M. and Wood, F. D. (1959) Studies of polyarthritis and other lesions induced in rats by injections of mycobacterial adjuvant: 1. General clinical and pathological characteristics and some modifying factors. *Arthritis Rheum.* **2**, 440.
15. Tokunaga, T., Yamamoto, H., Abe, H., et al. (1984) Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG.I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer. Inst.* **72**, 955–962.
16. Hemmi, H., Takeuchi, O., Kawai, T., et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740.
17. Albani, S., Keystone, E. C., Nelson, J. L. et al. (1995) Positive selection in autoimmunity: abnormal immune responses to a bacterial dnaJ antigenic determinant in patients with early rheumatoid arthritis. *Nat. Med.* **5**, 448–452.

Effects of Immunostimulatory DNA Oligonucleotides on Experimental Colitis

Daniel Rachmilewitz, Fanny Karmeli,
Leonor Leider-Trejo, Kenji Takabayashi,
Tomoko Hayashi, and Eyal Raz

1. INTRODUCTION

The etiology of human inflammatory bowel disease (IBD) is not yet known, and its pathogenesis is also poorly understood. At present, an uncontrolled or downregulated cellular immune response to an unknown trigger seems to play an important role. CD4⁺ T-cells were suggested to have a central role in the pathogenesis of experimental colitis (1) and human IBD, since in the latter, the CD4⁺ T cell pool is expanded both in the peripheral blood and in the inflamed mucosa (2). The balance between the three subsets of CD4⁺ T cells, which is well regulated under normal circumstances, is interrupted in several disease states. Human Crohn's disease (CD) is thought to be characterized by Th-1 response, which produces IL-2, IFN- γ , and tumor necrosis factor (TNF- α). Ulcerative colitis (UC) is dominated by Th-2 response, which produce antiinflammatory cytokines, such as; IL-4, IL-5, and IL-10. Models of experimental colitis also vary according to the dominant phenotype of Th-1 or Th-2 response. Spontaneous colitis in IL-10 knockout (KO) mice is mediated predominantly by Th-1 response (3). Tri or dinitrobenzene sulphonic acid induced colitis is characterized by predominant Th-2 response, and in this respect mimics UC (4). Administration of dextran sodium sulphate (DSS) to immune competent mice induce acute and chronic colitis, with features characteristic of a mixed Th-1/Th-2 response (1).

Up until recently, the conventional treatment of IBD included antiinflammatory drugs such as the aminosalicylates and immunosuppressive agents such as corticosteroids, azathioprine, cyclosporine, and methotrex-

ate. Lately, specific anti-TNF antibodies were added to the arsenal of drugs. These agents were shown to be effective in CD and are now being evaluated in UC.

Immunostimulatory DNA sequences (ISS) and their synthetic oligonucleotide (ODN) analogs have a biased stimulating effect on the mammalian innate immune system (5). In view of their induction of the secretion of Th-1 like cytokines (IL-12 and IFNs), their regulation of cell surface molecules involved in T-cell activation, and their antiapoptotic properties (6), it was logical to evaluate their possible modulation of experimental colitis. It was speculated that the continuation of the immunostimulatory effects on the innate immunity, together with antiinflammatory and antiapoptotic effects, might prevent and decrease the extent of colonic mucosal damage present in models of experimental colitis, as well as in human IBD. Amelioration of the mucosal damage could also limit the exposure of the involved mucosal tissue to luminal bacteria, thus further decreasing the perpetuation of the inflammatory cascade. If correct, the synergism between the various effects of immunostimulatory DNA, may be a novel and useful tool to prevent and treat IBD, whose current treatment is far from being satisfactory.

In this chapter the modulation of models of experimental colitis by ISS (7,8) and their effect on cytokine generation by human colonic mucosa of normal subjects and IBD patients, will be presented and discussed.

2. EFFECT OF ISS IN DSS INDUCED COLITIS

Colitis was induced in mice by the addition of 2.5% DSS to the drinking water, *ad libitum*. Control mice received tap water. Groups of mice were injected subcutaneously (s.c.) with ISS or mutated M-ODN 10–100 µg/animal, two hours prior to the addition of DSS to the drinking water. Other groups were given i.g. ISS 30–100 µg/animal, prior to DSS administration. All mice were weighed and inspected for diarrhea and rectal bleeding. Mice were sacrificed 7 d after treatment with DSS. The entire colon was dissected and weighed. Disease activity index (DAI) was determined by scoring changes in body weight and gross rectal bleeding. The DAI is the combined score of weight loss and bleeding. Scores were defined as follows: Changes in body weight; no loss = 0, 5 to 10% = 1, 10 – 15% = 2, 15 – 20% = 3, > 20% = 4. Hemocult; 0 = no blood, 2 = positive, 4 = gross blood. Mucosal samples were processed for determination of myeloperoxidase (MPO) activity and sections were fixed in formalin for histology.

Addition of DSS to the drinking water resulted in significant decrease in body weight and induced rectal bleeding. Colonic weight increased significantly by 59%, as compared to mice treated with tap water. MPO activity was also significantly increased in DSS treated mice.

Table 1
Effect of ISS and M-ODNs on DSS Induced Colitis

Treatment	No	Body weight (% decrease)	Disease activity (score)	Colonic weight (mg/10cm)	MPO (μ /g)
NONE	30	15.7 ± 1.2	5.5 ± 0.4	340 ± 15	4.2 ± 0.3
ISS	7	$1.2 \pm 0.4^*$	$0.9 \pm 0.4^*$	$248 \pm 15^*$	$1.7 \pm 0.4^*$
M-ODN	6	9.3 ± 2.0	3.5 ± 0.5	312 ± 0.3	3.6 ± 0.7

Mice were injected s.c. with a single dose of ISS or M-ODN (10 μ g) two h prior to colitis induction by DSS (2.5% in drinking water).

Data represent mean $\pm$ SEM.

*Significantly different from no treatment $p < 0.05$.

3. ISS-ODNS PREVENT AND AMELIORATE DSS INDUCED COLITIS

At first, the protective effect of a single administration of various ISS-ODNs or mutated M-ODNs on DSS induced colitis was evaluated. All 5 ISS-ODNs tested, significantly decreased the DAI, whereas the M-ODNs tested did not have a significant effect. The minimal effective dose was found to be 10 μ g/animal and 30 μ g/animal for s.c. and i.g. administration, respectively. Based on the initial screening, ISS-ODN (5'-TGACTGTGAACGTTCCGAGATGA-3') and the mutated (M)-ODN (5'-TGACTGTGTGTTCCCTTAGAGATGA-3') were selected for the subsequent studies.

ISS significantly decreased the DAI, the percent decrease in body weight and MPO activity, when compared to their respective levels in DSS treated mice. M-ODN administration did not affect these parameters, which were similar to their level in DSS treated mice (Table 1). Histologically, the mean histological score in ISS, plus DSS treated mice -6.6 ± 1.8 was 33% lower than the histological score in DSS treated mice -9.9 ± 0.6 . ISS was found not only to prevent, but also to reverse DSS induced colitis. When administered 48 h after induction of colitis, protective effects were observed similar to those observed in ISS pretreated animals. Intra-gastric administration of ISS two h prior to DSS administration was also found to effectively decrease the extent of DSS induced colonic damage (8).

4. ISS INHIBIT DSS INDUCED CELL DEATH AND DSS INDUCED INFLAMMATION

The damaged mucosal barrier observed in experimental colitis enhances the magnitude of the dysregulated inflammatory/immune response. To

Table 2
Effect of ISS and M-ODNs on Colonic
Caspase 3-like Activity
in DSS Treated Mice

Treatment	Caspase activity (RFU)
NONE	317 ± 20
DSS	496 ± 56
DSS/M-ODN	473 ± 64
DSS/ISS-ODN	200 ± 42*

Caspase-3-like activity in colonic tissue was assayed in lysates obtained from colonic tissues and was expressed as relative fluorescence units (RFU). Data represent mean ± SEM of four mice per group. *Denotes $p < 0.05$ in comparison to DSS- or M-ODN+DSS-treated mice (Students' *t*-test).

evaluate whether ISS enhances mucosal restitution, the extent of colonic epithelial cell death in ISS treated mice was evaluated. Colonic architecture was preserved and cellular infiltration was markedly reduced in DSS+ISS treated mice. These findings were consistent with TUNEL assay, which was negative in ISS-treated mice and markedly positive in the control groups (8).

The level of caspase-3-like activity in colonic tissue of ISS-treated mice was also significantly reduced (Table 2). The protective effect of ISS from DSS induced apoptosis was also evident in experiments conducted with cultured bone marrow derived macrophages (BMDM) that were pretreated with ISS for 2 h and then cultured with DSS for 2 d. The levels of caspase-3 and -9-like activities in the lysates of DSS+ISS-treated BMDM were markedly reduced in comparison to lysates from DSS or DSS+M-ODN treated BMDM, but similar to the levels obtained from lysates of BMDM treated with the apoptosis inhibitor, zVAD-fmk (8).

Messenger RNA levels of several inflammatory mediators in the affected distal colon were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) seven days after DSS treatment to mice treated with ISS and compared to those obtained from the unaffected duodenum (8). ISS markedly reduced the mRNA levels of inflammatory cytokines (IL-1 β , IL-6, and TNF- α), chemokines (KC, MIP-2, and MCP-2), and matrix metalloproteinases (MMP-1, MMP-3, MMP-9, and MMP-10). No induction of proinflammatory cytokines, chemokines or MMPs was observed in the unaffected duodenum. These observations indicate the antiinflammatory properties of ISS.

Table 3
Effect of ISS and M-ODNs on DNBS Induced Colitis

Treatment	Mortality	DAI	Weight (%)	MPO (u/g)	H.S.
NONE	4/10	1.8 ± 0.4	10.5 ± 2.5	1.8 ± 0.2	7.4 ± 1.5
ISS-ODN	0/10*	0.6 ± 0.3*	1.6 ± 0.7*	1.0 ± 0.1*	2.1 ± 0.5*
M-ODN	4/15	1.1 ± 0.09***	7.3 ± 0.6***	2.5 ± 0.3*	-

ISS-ODN was injected s.c. 2 h prior to induction of colitis by DNBS.

Results are mean ± SEM of 10 mice (Balb/c) per group.

DAI – disease activity index; Weight (%) - % decrease in body weight; MPO – myeloperoxidase (u/gr wet weight); HS- histological score.

*Denotes $p < 0.05$ of ISS-ODN-treated group in comparison to DNBS group (Chi Square and Students' t -test).

**vs ISS-ODN $p < 0.05$.

5. DNBS-INDUCED COLITIS

Colitis was induced in Balb/c mice by rectal instillation of 2,4,6-dinitrobenzene sulfonic acid 1mg/mouse, dissolved in 0.1ml of 50% ethanol. Treated animals received s.c. ISS or M-ODNS (10 µg/animal), two hours prior to induction of colitis. Mice were sacrificed seven days after DNBS administration. Disease activity index and MPO activity were determined as in DSS-induced colitis. Mice treated s.c. with ISS 10 µg/animal were fully protected from the mortality observed in mice treated with DNBS only. When compared to their surviving controls, ISS and DNBS treated mice had a lower DAI, significantly lower decrease in body weight and significantly lower level of colonic MPO activity, indicating the protective effect of ISS on DNBS induced colitis. The mean histological score in ISS+DNBS treated mice was also significantly lower than in mice treated with DNBS only (Table 3).

6. EFFECT OF ISS ON COLITIS IN IL-10 KO MICE

Six-week-old, female IL-10 KO mice (C57BL/6-IL-10 km1Cgn, Jackson Laboratory, Bar Harbor, ME) were weighed weekly and followed for the development of diarrhea, rectal bleeding, and rectal prolapse. Mice were treated s.c. once a week with 0.2 mL saline, ISS or M-ODN (10 µg/animal). Treatment started when mice were 10-wk-old, when there was no diarrhea, rectal bleeding or prolapse and continued for 4 weeks until sacrifice when the entire colon was resected, measured, and weighed. Sections from the distal colon were taken for histology and the rest of the entire colon was processed for determination of MPO activity. The body weight of all treatment groups was stable and similar in the control and the ODNs treated

Table 4
Effect of ISS and M-ODNs on Spontaneous Colitis in IL-10 KO Mice

Strain	Treatment	No	Rectal Prolapse	Colonic weight (mg/10 cm)	MPO (u/gr)	H.S.
WT	NONE	10	0	202±6*	0.7±0.1*	0
IL-10 KO	NONE	13	11	502±42	1.1±0.1	7.3±1.1
IL-10 KO	ISS	9	2*	341±32***	0.7±0.08***	1.8±1.1***
IL-10 KO	M-ODN	8	4	439±28	1.5±0.2	5.8±1.3

IL-10 KO mice were treated s.c. 1x/wk from wk 10–wk 14 with ISS or M-ODN (10µg). Mice were sacrificed after four weeks of treatment: the colon was isolated and weighed; mucosal MPO activity and Histological Score (H.S.) were determined.

Results are mean ± SEM.

*Significantly different vs NONE. **vs M-ODN (*p* < 0.05)

groups during the four weeks of the experiment (wk 10–wk 14). In the control, saline treated mice, rectal prolapse was observed on wk 12 in 40% of the animals and its incidence increased with time reaching 85% on wk 14. In contrast, in the ISS treated group, rectal prolapse was first noticed on week 14 affecting only 22% of the group. In M-ODN treated mice, rectal prolapse was already noticed in wk 12 in 50% of the mice, and its incidence did not increase thereafter. The amelioration of the spontaneous colitis in the ISS treated IL-10 KO mice was also reflected in the significant lower colonic weight, lower MPO activity, and the significant lower histological score when compared with the respective results in M-ODN or saline treated mice (Table 4).

7. EFFECT OF ISS ON HUMAN COLONIC CYTOKINE GENERATION

To assess whether the effects of ISS on experimental colitis are also applicable to human IBD, colonic biopsies were obtained from normal subjects and from IBD patients. The tissue was organ cultured for 24 h in the presence and absence of ISS or M-ODN and the generation of TNF-α, and IL-1β was determined. ISS was found to effectively decrease the upregulated IL-1β and TNF-α generation, by colonic mucosa of patients with active IBD. M-ODN had no significant effect on the generation of human colonic cytokines (Table 5).

8. DISCUSSION

The immunostimulatory properties of ISS, i.e., the secretion of cytokines such as IL-12 and IFNs, and the induction of costimulatory molecules such as; B7 and CD40 (5,9), were proposed to mediate induction of Th-1 response

Table 5
Effect of ISS or M-ODNs on Human Colonic IL-1 β and TNF- α Generation

	Spontaneous production ^b		% Change ^c Treatment		
	N ^a	(pg/mg wet wt)	None	ISS	M-ODN
IL-1 β					
NS	13	8.7 $\pm$ 3.2	100	47 $\pm$ 11 ^e	Not tested
UC	11	22.4 $\pm$ 3.5 ^d	100	53 $\pm$ 9 ^e	95 $\pm$ 20 ^f
CD	6	65.3 $\pm$ 16.0 ^d	100	66 $\pm$ 11 ^e	91 $\pm$ 29 ^f
TNF- α					
NS	13	2.9 $\pm$ 0.7	100	47 $\pm$ 11 ^e	Not tested
UC	8	24.3 $\pm$ 7.2 ^d	100	48 $\pm$ 10 ^e	156 $\pm$ 39 ^f
CD	6	32.0 $\pm$ 16.0 ^d	100	54 $\pm$ 16 ^e	122 $\pm$ 60 ^f

Colonoscopic biopsies were obtained from normal subjects (NS) and patients with active colitis (UC and CD) and organ cultured for 24 h in the presence and absence of ODN (10 μ g/mL). Human (h) IL-1 β and TNF- α in the medium were determined by ELISA. Results are mean $\pm$ SEM.

^aNumber of patients.

^bSpecimens were cultured in ODN free medium.

^cCytokine generation in ODN free medium was regarded as 100%.

^dSignificantly different from cytokine generation by normal colonic mucosa ($p < 0.05$).

^eSignificantly different from ODN free medium or M-ODN ($p < 0.05$).

^fSignificantly different from ISS $P < 0.05$.

to DNA (10), to inhibit allergic response (11) and to cross prime CTL responses (12). ISS also provides anti-apoptotic effects (6) and as was demonstrated in the current study, was associated with inhibition of caspase 3 and caspase 9 activation. These immunostimulatory and anti-apoptotic properties of ISS could also be beneficial for modulation of colonic inflammation in models of experimental colitis. Indeed, ISS was found to also reduce the frequency of DSS induced TUNEL positive cells in the colonic epithelium, and DSS induced caspase 3 activity in colonic tissue (8). Furthermore, ISS was found to not only decrease the extent and severity of DSS induced colitis, which is characterized by a mixed Th-1/Th-2 response (1), but also to affect DNBS/TNBS colitis, which is characterized by a Th-2 response (4), and to downregulate spontaneous colitis in IL-10 KO mice characterized by a Th-1 response (3).

The protection provided by ISS in IL-10 KO mice spontaneous colitis, despite its Th-1 adjuvanticity, underscore the important contribution of the antiapoptotic and antiinflammatory effects of ISS, regardless of the nature

of the dysregulated CD4⁺ response in these models. These observations suggest that the dysregulated CD4⁺ response in the models of experimental colitis is not the primary inducer of the mucosal inflammation.

ISS was found to activate NF- κ B (13) and to induce the transcription of Bcl-X₂ (14), which probably synergize with the ISS activated DNA repair enzyme DNA-PK₈ (15) to inhibit apoptosis of mucosal epithelial cells. The protective effects of ISS probably decrease the mucosal barrier dysfunction induced by DSS or DNBS and the invasion of commensurate bacteria that further perpetuate mucosal inflammation. As shown in this study, ISS decreased colonic mRNA levels of several proinflammatory mediators such as; IL-1 β and TNF- α . ISS also inhibited mRNA levels of the chemokines KC, MCP-2 and MIP-2, which may explain the reduced colonic neutrophil infiltration and reduced MPO activity.

The present study also demonstrates that the amelioration of colonic inflammation in models of colitis may be applicable to the human. ISS was found to effectively decrease the upregulated generation of IL-1 β (16) and TNF- α (17), by inflamed colonic mucosa of patients with active IBD. In light of the beneficial therapeutic effects of anti-TNF- α antibodies in patients with refractory Crohn's disease (18), it is clear that TNF- α plays a pivotal role. It therefore seems that ISS induced inhibition of TNF- α generation by itself, and definitely in synergism with its other antiapoptotic and immunostimulatory effect, may be of great value in the treatment of IBD.

ACKNOWLEDGMENTS

Supported by the Foundation for Prevention and Treatment of G.I. Disorders (DR) and NIH grant AI-40682 (ER).

REFERENCES

1. Blumberg, R. S., Saubermann, L. J. and Strober, W. (1999) Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr. Opin. in Immunol.* **11**, 648–56.
2. Sartor, R. B. (1997) Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am. J. Gastroenterol.*, **92**, 5S–11S.
3. Davidson, N. J., Fort, M. M., Muller, W., Leach, M. W., and Rennick, D. M. (2000) Chronic colitis in IL-10^{-/-} mice: insufficient counter regulation of a Th1 response. *Int. Rev. Immunol.*, **19**, 91–121.
4. Dohi, T., Fujihashi, K., Rennert, P. D., Iwatani, K., Kiyono, H., and McGhee, J. R. (1999) Hapten-induced colitis is associated with colonic patch hypertrophy and T helper cell 2-type responses. *J. Exp. Med.* **189**, 1169–1180.
5. Martin-Orozco, E., Kobayashi, H., Van Uden, J., Nguyen, M. D., Kornbluth, R. S., and Raz, E. (1999) Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118.

6. Krieg, A. M. and Yi, A. K. (2000) Rescue of B cells from apoptosis by immune stimulatory CpG DNA. *Springer Semin. Immunopathol.* **22**, 55–61.
7. Rachmilewitz, D., Karmeli, F., Raz, E. (2000) Immunostimulatory DNA sequences ameliorate the extent of experimental colitis in mice and rats. *Gastroenterology* **118**, A576.
8. Rachmilewitz, D., Karmeli, F., Takabayashi, K., et al. (2002). Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* **122**, 1428–1441.
9. Bauer, M., Heeg, K., Wagner, H., and Lipford, G. B. (1999) DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology* **97**, 699–705.
10. Sato, Y., Roman, M., Tighe, H., et al. (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.
11. Horner, A. A., Van Uden, J. H., Zubeldia JM, Broide D, Raz E. (2001) DNA-based immunotherapeutics for the treatment of allergic disease. *Immunological Reviews* **179**, 102–118.
12. Cho, H. J., Takabayashi, K., Cheng, P. M., et al. (2000) Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nature Biotechnol.* **18**, 509–514.
13. Stacey, K. J., Sester, D. P., Sweet, M. J., and Hume, D. A. (2000) Macrophage activation by immunostimulatory DNA. *Curr. Top. Microbiol. Immunol.* **247**, 41–58.
14. Yi, A. K., Hornbeck, P., Lafrenz, D. E., and Krieg, A. M. (1966). CpG DNA rescue of murine B lymphoma cells from anti-IgM-induced growth arrest and programmed cell death is associated with increased expression of c-myc and bcl-xL. *J. Immunol.* **157**, 4918–4925.
15. Chu, W. M., Gong, X., Li, Z. W., et al. (2000) DNA-PKcs is required for activation of innate immunity by immunostimulatory DNA. *Cell* **103**, 909–918.
16. Ligumsky, M., Simon, P. L., Karmeli, F., and Rachmilewitz, D. (1990) Role of interleukin 1 in inflammatory bowel disease—enhanced production during active disease. *Gut* **31**, 686–689.
17. Van Deventer, S. J. (1997) Tumor necrosis factor and Crohn's disease. *Gut* **40**, 443–448.
18. Targan, S. R., Hanauer, S. B., van Deventer, S. J., et al. (1997) A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N. Engl. J. Med.* **337**, 1029–1035

PART IX

SAFETY CONSIDERATIONS

CpG-ODN–Safety Considerations

Daniela Verthelyi and Dennis M Klinman

1. INTRODUCTION

Bacterial DNA and synthetic oligonucleotides (ODN) containing unmethylated cytidine-phosphate guanosine dinucleotide (CpG) motifs (CpG-ODN) stimulate a rapid innate immune response and condition the development of adaptive immunity. Although the therapeutic applications of CpG DNA have been studied extensively in species ranging from fish to man, their long-term in vivo safety is less well characterized.

CpG-ODN could potentially cause harm to the host

1. Through direct inflammatory or toxic effects (either alone or in combination with other agents).
2. By inducing a persistent alteration in the immune milieu by skewing the Th1:Th2 cytokine balance.
3. By contributing to the development of autoimmune disease (1,2).

This work reviews current information pertaining to each of these issues.

The overwhelming majority of preclinical and clinical studies involving CpG ODN utilize DNA composed of nuclease resistant phosphorothioate (PS) rather than natural phosphodiester (PO) bases. The former have a longer half-life and superior activity in vivo (3). This review will focus on the effects of PS ODN, separating where possible the sequence-specific toxicity of CpG motifs from the broader toxicity of high-dose PS oligonucleotides.

2. RESULTS

2.1. *Inflammatory and Toxic Effects of CpG ODN*

2.1.1. *Local Inflammation*

Immune adjuvants boost the local production of cytokines and chemokines, thereby improving the recruitment of immunologically active cells. Yet, the clinical use of powerful Th1 adjuvants (such as Complete Freund's adjuvant) has been limited owing to their induction of excessive local inflammation. It was therefore relevant to explore the level of inflammation induced by CpG ODN.

CpG ODN administered at 1–2 mg/kg IM trigger a local inflammatory process, characterized by up to a 40-fold increase in the number of macrophages, granulocytes, and dendritic cells infiltrating the site of injection and draining lymph nodes. This is accompanied by a 6–13 fold increase in the production of mRNA encoding chemokines such as MIP-1 α , MIP-1 β , and RANTES (4). When CpG ODN are delivered intratracheally, local inflammation is again observed, as manifest by the accumulation of neutrophils and increased levels of TNF α and IL-6 in bronchiolar lavage fluid (BALF) (5). Thus, there is reason to be concerned with the local reactogenicity of CpG ODN. Despite this, a recent study compared the relative potency of various immune adjuvants. Results indicate that CpG DNA elicits less local inflammation than other adjuvants (such as Titermax, complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA), [6]). In addition, recently released data from a phase I/II clinical study indicate that administration of CpG ODN (7 μ g/kg) is well tolerated systemically and locally by healthy adults ($N = 20$, [7]). Further studies will establish whether higher doses will be equally well tolerated.

2.1.2. *Systemic Toxicity*

The use of ODN for antisense therapy of cancer and other diseases mandated that the pharmacokinetics and toxicity of PS ODN be determined. PS ODN at high doses (≥ 10 mg/kg) cause spleen and lymph node hyperplasia, and the infiltration of multiple organs by mononuclear cells (3,8). Doses in excess of 10 mg/kg can cause complement activation, increase serum levels of activated partial thromboplastin and renal tubular degeneration in mice and primates (3,9). Although these effects are sequence independent, doses as low as 1 mg/kg of PS CpG ODN can cause splenomegaly (10). This effect peaks 6 d after ODN administration, and is associated with extramedullary hematopoiesis and the accumulation of granulocytes and monocyte precursors in the spleen (10). Splenomegaly, appears to reflect the synergy between

Table 1
Health Assessment of Mice One Month After Ten Weekly Doses of CpG ODN

Parameter measured	PBS control	CpG-ODN treated
Body weight (grams)	21.12 ± 0.38	21.18 ± 0.48
Liver weight (grams)	0.97 ± 0.08	1.01 ± 0.05
Spleen size (# cells × 10 ⁻⁶)	85.60 ± 14.38	91.60 ± 10.16

BALB/c mice were injected ip with PBS or 2 mg/kg of CpG-ODN for 10 successive weeks. One month after the last injection, animals were sacrificed. Total body and liver weight were recorded, as were the number of white blood cells in the spleen. Data represent the mean ± SD of six individually tested mice/group from two different experiments.

immune activation by CpG motif plus lymphoid hyperplasia induced by PS ODN. Of note, extramedullary hematopoiesis is frequent in mice but are rare in humans, and has not been reported in primates treated with ODN.

Our lab examined the toxicity of repeatedly administering 1–2 mg/kg of PS CpG ODN (a physiologically active dose) to BALB/c mice (11,12). Animals injected weekly or biweekly for up to 4 mo remained physically vigorous. None became sick, lost weight, or died, nor did they develop proteinuria or other clinical manifestations of autoimmune disease (*N* > 200). Peripheral blood smears were normal throughout the study. Polyclonal B-cell activation was observed in many animals treated weekly with CpG ODN, and this was associated with a modest increase in serum IgM levels. However, these effects waned by 30 d after the cessation of therapy (12). Histologic analysis of organs from mice treated with CpG ODN for 10 wk (including, spleen, lymph nodes, muscle, intestine, heart, lung, adrenal gland, kidney, and liver) revealed neither macroscopic nor microscopic evidence of tissue damage or inflammation (Table 1).

Although CpG ODN at therapeutically active doses were nontoxic, Cowdery et al. showed that mice treated with bacterial DNA (1–10 mg/kg) followed by an otherwise sublethal dose of lipopolysaccharide (LPS) suffered a 75% mortality associated with a 3- and 10-fold increase in serum TNFα and IL-6 levels, respectively (13). In a separate study, mice presensitized with D-galactosamine and then given CpG ODN (2 μg/kg) also developed TNFα-mediated toxic shock (13–15). These studies suggest that CpG ODN alone, are not directly toxic, but that the Th1 polarization of the immune system they elicit may synergize with other microbial products to cause illness or death.

Most of the safety studies of CpG ODN have been performed in rodents. Unfortunately, those CpG motifs that are optimally active in mice are rela-

Table 2
Cytokine Secretion by PBMC from SLE Patients and Healthy Controls Stimulated with CpG-ODN

	SLE patients		Controls	
	Baseline	CpG	Baseline	CpG
IL-6 (K ODN)	127±16	196±19	198±26	1526±138
IFNγ (D ODN)	11±1	25±4	18±3	202±31

Response of PBMC from normal volunteers (*n* = 12) and SLE-patients (*n* = 12) to CpG-stimulus. Cytokine production was evaluated by ELISA from supernatant of 72-h cultures. Geometric mean ± SEM.

tively ineffective at stimulating human peripheral blood mononuclear cells (PBMC) (16,17). Moreover, there are differences in the nature and magnitude of the immune response elicited by CpG DNA in rodents vs primates. Work in our lab indicates that two structurally distinct types of CpG motif (referred to as “K” and “D”) stimulate primate PBMC. “D” ODN preferentially activate natural killer (NK) cells to produce IFNγ, whereas “K” ODN induce B-cell proliferation and the production of IL-6 and IgM by monocytes and B cells (Table 2) (17).

To evaluate the safety of CpG ODN in primates, “K” and “D” ODN were administered to rhesus macaques. PBMC from humans and macaques respond similarly to these motifs when stimulated in vitro. Five animals/group were injected ID twice with 100 μg/kg of ODN. This dose was previously shown to be immunogenic in rhesus macaques (Verthelyi, unpublished observations). All of the animals remained healthy. There were no hematologic or serologic abnormalities 3 d after injection and no weight loss or change in behavior detected during 4 mo of follow up (Table 3 and data not shown). To determine whether CpG ODN induced acute toxicity, an additional group of macaques was injected with 1 mg/kg of “K”, “D” or control ODN. One day later, the animals were sacrificed and a thorough histologic evaluation conducted. None of the animals had gross or microscopic changes in any tissue examined, nor was there evidence of lymphadenopathy, splenomegaly or hepatomegaly (Klinman, unpublished observations). Although more thorough studies should be performed, these studies of non-human primates support results from mice indicating that PS CpG ODN can be used safely at doses that are therapeutically active.

2.2. Effect of CpG-ODN on Immune Homeostasis

CpG-ODN stimulate a strong Th1 dominated immune response characterized by the production of IFNγ and IL-12 by lymphocytes and macrophages (16–19). This Th1-driven response may be harnessed to improve the

Table 3
Hematologic Assessment of Rhesus Macaques Treated with CpG DNA

		WBC	RBC	PLT
D” ODN	PRE Rx	5.7 ± 2.2	5.2 ± 0.4	473±85
	Post Rx	8.2 ± 2.2	4.5 ± 0.2	388±82
K ODN	PRE Rx	4.9 ± 2.2	5.3 ± 0.4	398±168
	Post Rx	6.6 ± 2.2	4.3 ± 0.3	353±83
CONTROL ODN	PRE Rx	5.8 ± 1.3	5.1 ± 0	470±59
	Post Rx	7.6 ± 2.0	4.3 ± 0.2	392±26
SALINE	PRE Rx	8.7 ± 2.4	4.9 ± 0.2	498±68
	Post Rx	9.4 ± 3.5	4 ± 0.2	420±88

Twenty 2-yr-old Rhesus macaques were injected ID with 100µg/kg of each ODN. Full chemistry and hematology panels were run on peripheral blood immediately before and three days after treatment. Data is expressed in thousands. No significant differences were evident after CpG ODN administration.

activity of protein and DNA-based vaccines and prevent the development of Th2-dependent allergic responses responses (20–22). This Th1 bias persists for up to 6 weeks, consistent with the ability of CpG-ODN to induce long-term antigen-specific skewing of the immune milieu (22–24).

A bias toward Th1 immunity may help the host resist infection by intracellular pathogens (such as *Leishmania major*, *Trypanosoma cruzi* or *Mycobacterium tuberculosis*) or to desensitize patients with allergy (25–28). However, it could be detrimental when the host needs to mount a Th2 response against other pathogens, such as nematodes or helminths (29–31). It could also harm individuals predisposed to develop Th1 mediated autoimmune disease (see **Subheading 2.3, 32,33**).

To determine whether in vivo administration of CpG-ODN can permanently alter the cytokine milieu, BALB/c mice were injected weekly for 10 wk with 1–2 mg/kg of CpG ODN. At 1 and 5 d posttreatment, the number of spleen cells stimulated to secrete Th1 cytokines was significantly increased in treated versus control mice. However, this effect did not persist when therapy was discontinued. Thirty days after the final injection, the number of cells spontaneously secreting IL-4 and IFNγ was similar in the CpG DNA and saline treated groups (12). Another study monitored the effect of injecting CpG ODN into the right hind foot of BALB/c mice. CpG ODN induced lymphadenopathy in the draining lymph nodes that lasted 14 d, with cells in

these lymph nodes secreting higher levels of IFN γ and IL-12 for approximately 1 wk after treatment (18). However, neither of these effects persisted long term. Moreover, the CpG ODN did not affect immune reactivity systemically, since the cellularity and the pattern of cytokine secretion in the contra lateral lymph nodes was similar to that of untreated controls (18).

These results suggest that CpG ODN can alter the balance between Th1:Th2 cytokine secreting cells. However, this effect is transitory and tends to be local rather than systemic, thereby reducing the likelihood of long-term detrimental effects to the host. In this context, there is no preclinical or clinical evidence that CpG ODN cause long-term antigen nonspecific skewing of the immune repertoire.

2.3. Capacity of CpG ODN to Cause Autoimmune Disease

CpG DNA stimulates polyclonal B-cell activation, triggers the production of proinflammatory cytokines, accelerates the maturation of professional antigen presenting cells, and prevents the apoptotic death of activated immune cells (34–36). In various model systems, each of these effects has been associated with the development of autoimmune disease.

2.3.1. CpG DNA and Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease of multifactorial origin, with environmental and genetic factors combining to cause illness. CpG motifs present in bacterial DNA may play a role in lupus pathogenesis because

1. Lupus flares are frequently preceded by microbial infections.
2. CpG DNA can exacerbate the production of anti-DNA autoantibodies (a prominent feature of SLE) both by supplying cross-reactive antigen and by triggering polyclonal B-cell activation.
3. Chloroquine, an antimalarial drug used effectively to treat SLE, also blocks the activity of CpG ODN by interfering with endosomal acidification, a step necessary for CpG ODN intracellular signaling (37–42).

To evaluate the ability of CpG motifs to induce or accelerate the development of systemic autoimmunity, the classic NZB/W mouse model was used. Young NZB/W mice treated with bacterial DNA produced anti-DNA autoantibodies more rapidly and at higher titers than controls treated with mammalian DNA. Surprisingly, these mice developed less renal pathology and lived longer than control mice (43). Because SLE is characterized by an increased Th2:Th1 cytokine ratio (44), the protective effect of CpG-ODN may reflect a normalization of this imbalance through increased Th1 cytokine production, and a concomitant shift in the isotype of anti-DNA antibodies (43,45). Consistent with such a possibility, CpG-ODN treatment

Table 4
Preclinical Safety Studies of CpG ODN in Mice

Mice	Treatment	Evaluated	IL-6	IFN γ	IgG anti-DNA	
					Titer	Histology
NZB/W	Untreated	1 day	140 $\pm$ 38	21 $\pm$ 7	32 $\pm$ 4	
	CpG ODN	1 day	736 $\pm$ 32	48 $\pm$ 10	41 $\pm$ 5	
	CpG ODN	1 month	153 $\pm$ 25	28 $\pm$ 12	33 $\pm$ 6	
BALB/c	Untreated	1 day	314 $\pm$ 65	11 $\pm$ 3	1 $\pm$ 1	Normal
	CpG ODN	1 day	816 $\pm$ 130	22 $\pm$ 5	2 $\pm$ 1	Normal
	CpG ODN	1 month	288 $\pm$ 48	10 $\pm$ 4	1 $\pm$ 1	Normal

BALB/c or NZB/W mice were injected three or more times with 2 mg/kg of CpG ODN at 2–6 wk intervals. Cytokine secreting spleen cells from these animals were monitored by ELISpot assay, while serum IgG anti-DNA autoantibody titers were determined by ELISA. Results reflect the average of at least three independently studied animals/group.

of NZB/W mice increased the number of IFN γ and IL-12 secreting cells (Table 4) while reducing the ratio of IgG1:IgG2a anti-DNA autoantibodies secreted in vivo (45).

Although animal studies indicate that the administration of CpG-DNA does not cause or exacerbate systemic autoimmune disease, data showing that lupus patient serum contains abnormally high levels of GC rich DNA prompted further study. Our lab examined whether PBMC from SLE patients responded abnormally to “K” or “D” ODN (17). Rather than hyper-responding to stimulation by CpG DNA, PBMC from SLE patients produced less IFN γ and IL-6 than did controls (Table 2). This hypo-responsiveness was not owing to ongoing treatment with corticosteroids or chloroquine, since patients with rheumatoid arthritis receiving the same drugs responded normally to CpG ODN stimulation. The abnormal cytokine response of lupus PBMC to CpG DNA may reflect either acclimation to chronic bacterial DNA stimulation, or efforts by the immune system of SLE patients to homeostatically reduce responsiveness to foreign stimuli. Thus, animal studies suggest that CpG DNA may blunt the development of SLE by promoting the secretion of Th1 cytokines, whereas studies of PBMC from patients fail to reveal any hyper-responsiveness to CpG DNA.

2.3.2. The Adjuvant Effect of CpG DNA may Contribute to the Development of Organ-Specific Autoimmunity

Organ-specific autoimmune diseases are frequently characterized by strong autoantigen-specific Th1 immune responses. Because CpG ODN

boost Th1 immunity, there is concern that exposure to CpG DNA may induce or worsen organ specific diseases. A useful model of Th1-dependent organ-specific autoimmunity is multiple sclerosis (MS), which can arise in a setting of infectious illness (46). Murine experimental autoimmune encephalomyelitis (EAE) provides a model of human MS (47,48). To examine whether CpG-DNA can contribute to the development of EAE, SJL mice were immunized with myelin basic protein 87–106 (MBP) plus ODN. Disease inducing effector cells were not elicited in this EAE resistant strain following treatment with MBP alone. However, the combination of MBP plus CpG-DNA caused disease, manifest by severe muscle weakness. This effect was linked to the upregulation of IL-12 production (47,48). CpG-ODN was not detrimental in other animal models of Th1 mediated autoimmune disease (49,50). For example, studies of insulin dependent diabetes mellitus (IDDM) showed that treatment of 8-wk-old NOD mice with CpG-ODN reduced the incidence of IDDM by 50% (51). In this model CpG-ODN induced the production of IL-10 which, the authors postulate may have arrested the autoimmune process.

Another mechanism by which CpG-ODN may contribute to the development of organ specific autoimmune disease is by enhancing the presentation of microbial antigens that mimic self. For example, Bachmaier et al. showed that combining chlamydia antigens with CpG (but not control) DNA boosted the production of heart muscle specific autoantibodies, resulting in the development of autoimmune myocarditis in mice (52). This effect was related to the ability of CpG DNA to increase autoantigen presentation and promote the production of proinflammatory cytokines.

These data suggest that the adjuvant effect of CpG-DNA may influence the development of organ-specific autoimmunity, either by changing the cytokine milieu or by promoting the presentation of self-antigens, or antigens crossreactive with self. This raises concerns regarding both the efficacy and safety of CpG-DNA in subjects predisposed to develop autoimmune disease. Balancing these concerns is data showing that normal animals treated repeatedly with CpG-ODN do not develop autoimmune disease, and the absence of autoimmune sequella following CpG-DNA treatment of humans.

3. DISCUSSION

Since their discovery in 1995, considerable insight has been gained into the mode of action, specificity, and therapeutic potential of CpG DNA. Data from preclinical studies indicate that CpG-ODN are nontoxic at therapeutically active doses. Yet, they can cause illness or death when combined with other

immunomodulatory agents. Although CpG motifs support the development of proinflammatory and Th1 immune responses, these effects tend to be localized both spatially and temporally, reducing concern that long-term abnormalities in immune homeostasis will result from therapy with CpG containing products. There is little evidence that CpG-DNA induces or accelerates the development of systemic autoimmunity, but in several model systems, they did promote the development of organ specific autoimmune disease.

Several phase I clinical trials have been conducted to evaluate the activity of CpG-ODN as immune adjuvants, for cancer therapy, or to prevent allergy. The limited safety data released from these trials indicates that CpG DNA does not cause major systemic toxicity. Only through considerable additional research will the long-term safety and efficacy of these products be established.

REFERENCES

1. Goldberg, B. (2000) Beyond danger: unmethylated CpG dinucleotides and the immunopathogenesis of disease. *Immunol. Lett.* **73**, 13–18.
2. Klinman, D. M. (2000) Activation of the innate immune system by CpG oligodeoxynucleotides: immunoprotective activity and safety. *Springer Semin. Immunopathol.* **22**, 173–183.
3. Levin, A. (1999) A review of issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. *Biochim. Biophys. Acta.* **1489**, 68–84.
4. Takeshita, S., Takeshita, F., Haddad, D. E., Ishii, K. J. and Klinman, D. M. (2000) CpG oligodeoxynucleotides induce murine macrophages to upregulate chemokine mRNA expression. *Cell. Immunol.* **206**, 101–106.
5. Schwartz, D. A., Quinn, T. J., Thorne, P. S., Sayeed, S., Ae, Y. and Krieg, A. M. (1997) CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* **100**, 68–73.
6. Weeratna, R. D., McCluskie, M. J., Xu, Y. and Davis, H. L. (2000) CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* **18**, 1755–1762.
7. Davis, H. L. and Coley Pharmaceutical Group. (2000) CpG ODN is safe and highly effective in humans as adjuvant to HBV vaccine: preliminary results of Phase I trial with CpG ODN 7909. *Internet Communication*.
8. Agrawal, S. (1999) Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides. *Biochim. Biophys. Acta.* **1489**, 53–68.
9. Monteith, D. K., Horner, M. J., Gillett, N. A., et al. (1999) Evaluation of the renal effects of an antisense phosphorothioate ODN in monkeys. *Toxicol. Pathol.* **27**, 307–317.
10. Sparwasser, T., Hultner, L., Koch, E. S., Luz, A., Lipford, G. B. and Wagner, H. (1999) Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. *J. Immunol.* **162**, 2368–2374.

11. Elkins, K. L., Rhinehart-Jones, T. R., Stibitz, S., Conover, J. S. and Klinman, D. M. (1999) Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J. Immunol.* **162**, 2991–2998.
12. Klinman, D. M., Conover, J. and Coban, C. (1999) Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect. Immun.* **67**, 5658–5663.
13. Cowdery, J. S., Chace, J. H., Yi, A.-K. and Krieg, A. M. (1996) Bacterial DNA induces NK cells to produce IFN γ in vivo and increases the toxicity of lipopolysaccharides. *J. Immunology* **156**, 4570–4575.
14. Sparwasser, T., Meithke, T., Lipford, G., et al. (1997) Bacterial DNA causes septic shock. *Nature* **386**, 336–338.
15. Lipford, G. B., Sparwasser, T., Bauer, M., Zimmermann, S., Koch, E., Heeg, K.I. and Wagner, H. (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur. J. Immunol.* **27**, 3420–3426.
16. Bauer, M., Heeg, K., Wagner, H. and Lipford, G. B. (1999) DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology* **97**, 699–705.
17. Verthelyi, D., Ishii, K. J., Gursel, M. and Klinman, D. M. (2000) Two kinds of CpG DNA elicit distinct responses in human PBMC. *J. Immunol.* (In Press)
18. Lipford, G. B., Sparwasser, T., Zimmermann, S., Heeg, K. and Wagner, H. (2000) CpG-DNA-mediated transient lymphadenopathy is associated with a state of Th1 predisposition to antigen-driven responses. *J. Immunol.* **165**, 1228–1235.
19. Klinman, D. M., Yi, A., Beaucage, S. L., Conover, J. and Krieg, A. M. (1996) CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN γ . *Proc. Natl. Acad. Sci. USA* **93**, 2879–2883.
20. Klinman, D. M., Yamshchikov, G. and Ishigatsubo, Y. (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. *J. Immunol.* **158**, 3635–3642.
21. Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. and Harding, C. V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper (Th1) immunity. *J. Exp. Med.* **186**, 1623–1631.
22. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D. and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN- γ production in cells from allergic individuals and inhibit IgE synthesis in vitro. *Eur. J. Immunol.* **29**, 2344–2353.
23. Sur, S., Wild, J. S., Choudhury, B. K., Alam, R., Sur, N. and Klinman, D. M. (1999) Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* **162**, 6284–6291.
24. Serebrisky, D., Teper, A. A., Huang, C. K., et al. (2000) CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. **165**, 5906–5912.

25. Corral, R. S. and Petray, P. B. (2000) CpG DNA as a Th1-promoting adjuvant in immunization against *Trypanosoma cruzi*. *Vaccine* **19**, 234–242.
26. Zimmermann, S., Egeter, O., Hausmann, S., et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine Leishmaniasis. *J. Immunol.* **160**, 3627–3630.
27. Freidag, B. L., Melton, G. B., Collins, F., et al. CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of *Mycobacterium bovis* BCG vaccination in mice challenged with *M. tuberculosis*. *Infect. Immun.* **68**, 2948–2953.
28. Walker, P. S., Scharton-Kersten, T., Krieg, A. M., et al. (1999) Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12 and IFN γ dependent mechanisms. *Proc. Natl. Acad. Sci. USA.* **96**, 6970–6975.
29. Svetic, A., Madden, K. B., Zhou, X. D., et al. (1993) A primary intestinal helminthic infection rapidly induces a gut-associated elevation of Th2-associated cytokines and IL-3. *J. Immunol.* **150**, 3434–3441.
30. Urban, J. F. J., Madden, K. B., Cheever, A. W., Trotta, P. P., Katona, I. M. and Finkelman, F. D. (1993) IFN inhibits inflammatory responses and protective immunity in mice infected with the nematode parasite, *Nippostrongylus brasiliensis*. *J. Immunol.* **151**, 7086–7094.
31. Urban, J. F. J., Madden, K. B., Svetic, A., et al. (1992) The importance of Th2 cytokines in protective immunity to nematodes. *Immunol. Rev.* **127**, 205–220.
32. Calcinaro, F., Gambelunghe, G. and Lafferty, K. J. (1997) Protection from autoimmune diabetes by adjuvant therapy in the non-obese diabetic mouse: the role of interleukin-4 and interleukin-10. *Immunol. Cell Biol.* **75**, 467–471.
33. Cameron, M. J., Arreaza, G. A., Zucker, P., et al. (1997) IL-4 prevents insulinitis and insulin-dependent diabetes mellitus in nonobese diabetic mice by potentiation of regulatory T helper-2 cell function. *J. Immunol.* **159**, 4686–4692.
34. Krieg, A. M.; Yi, A., Matson, S., et al. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–548.
35. Krieg, A. M. (2000) The role of CpG motifs in innate immunity. *Cur. Op. Immunol.* **12**, 35–43.
36. Krieg, A. M. (2000) Rescue of B cells from apoptosis by immune stimulatory CpG DNA. *Springer Semin. Immunopathol.* **22**, 55–61.
37. Sparwasser, T., Koch, E., Vabulas, R. M., et al. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054.
38. Krieg, A. M. (1995) CpG DNA : A pathogenic factor in systemic lupus erythematosus? *J. Clin. Immunol.* **15**, 284–292.
39. Mor, G., Singla, M., Steinberg, A. D., Hoffman, S. L., Okuda, K. and Klinman, D. M. (1997) Do DNA vaccines induce autoimmune disease? *Hum. Gene Ther.* **8**, 293–300.
40. Macfarlane, D. E. and Manzel, L. (1999) Immunostimulatory CpG-oligodeoxynucleotides induce a factor that inhibits macrophage adhesion. *J. Lab. Clin. Med.* **134**, 501–509.
41. Manzel, L. and Macfarlane, D. E. (1998) CpG-oligodeoxynucleotide supports growth of IL-6-dependent 7TD1 murine hybridoma cells. *Life Sci.* **62**, 23–27.

42. Macfarlane, D. E. and Manzel, L. (1998) Antagonism of immunostimulatory CpG-oligodeoxynucleotides by quinacrine, chloroquine, and structurally related compounds. *J. Immunol.* **160**, 1122–1131.
43. Gilkeson, G. S., Ruiz, P., Pippen, A. M., Alexander, A. L., Lefkowitz, J. B. and Pisetsky, D. S. (1996) Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J. Exp. Med.* **183**, 1389–1397.
44. Verthelyi, D. and Klinman, D. M. (1998) Impact of the type 1: type 2 cytosine imbalance on disease activity in SLE. In: *Lupus-Cellular and Molecular Pathogenesis*. (Kammer, G. M. and Tsokos, G. C., eds.) Humana Press, Totowa, NJ. pp. 361–370.
45. Gilkeson, G. S., Conover, J. S., Halpern, M., Pisetsky, D. S., Feagin, A. and Klinman, D. M. (1998) Effects of bacterial DNA on cytokine production by (NZB/NZW)F1 mice. *J. Immunol.* **161**, 3890–3895.
46. Casetta, I. and Garnieri, E. (2000) Clinical infections and multiple sclerosis: contribution from analytical epidemiology. *J. Neurovirol.* **s2**, 151.
47. Segal, B. M., Klinman, D. M. and Shevach, E. M. (1997) Microbial products induce autoimmune disease by an IL-12 dependent process. *J. Immunol.* **158**, 5087–5091.
48. Segal, B. M., Chang, J. T. and Shevach, E. M. CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells in vivo. *J. Immunol.* **164**, 5683–5688.
49. Boccaccio, G. L. (1999) Non-coding plasmid DNA induces IFN-gamma in vivo and suppresses autoimmune encephalomyelitis. *Int. Immunol.* **11**, 289–296.
50. Lobell, A. (1999) Presence of CpG DNA and the local cytokine milieu determine the efficacy of suppressive DNA vaccination in experimental autoimmune encephalomyelitis. *J. Immunol.* **163**, 4754–4762.
51. Quintana, F. J. (2000) Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J. Immunol.* **165**, 6148–6155.
52. Bachmaier, K., Meu, N., Maza, L. M., Pal, S., Nessel, A. and Penninger, J. M. (1999) Chlamydia infections and heart disease linked through antigenic mimicry. *Science* **283**, 1335–1339.

Index

A

Adjuvant arthritis, *see* Arthritis

Allergic conjunctivitis,

clinical features, 315, 316

conventional treatment,

drug therapy, 316

immunotherapy, 316

epidemiology, 315

immunostimulatory sequence

therapy,

dose-response and regimen

development, 320, 321

humoral response, 320

hypersensitivity scoring, 318

interferon- γ response, 319, 320

mechanism of action, 321–323

prospects for clinical use, 323,

324

rationale, 316, 317

mouse model, 317, 318

pathophysiology, 315, 316

Allergy, *see also* *Mycobacterium*

tuberculosis,

allergen–immunostimulatory

sequence conjugate therapy,

rationale, 280, 281

Th2 downregulation, 180–182,

185, 186, 281

clinical prospects of

immunomodulatory DNA,

283, 285

comparison of DNA vaccination

and immunostimulatory

sequence modulation, 282,
283

DNA immunomodulation

mechanisms, 281, 282

immunotherapy,

goals, 280

options, 279, 280

Th1 response protection in

animals, 156, 157, 171

Th2 response, 156, 157

Amba1–immunostimulatory

sequence conjugate, Th2

downregulation and allergy

therapy, 180–182, 185, 186,

295, 296

Antigen–immunostimulatory

sequence conjugates,

allergen–immunostimulatory

sequence conjugates and Th2

downregulation, 180–182,

185, 186

clinical applications, 185, 186

cytotoxic T lymphocyte response,

177, 179, 180, 184

dendritic cell activation

mechanisms, 183–185

gp120 conjugate immune

response, 179, 180, 185

humoral immunity, 177, 178, 180

ovalbumin–immunostimulatory

sequence conjugate,

effects on mouse asthma model,

183–185

tumor vaccine, 184

- preparation, 176, 177, 184
- rationale for use, 175, 176
- safety, 186
- Th1 response, 177, 180–182
- Antigen presentation, *see* Dendritic cell; Macrophage
- AP-1, activation by CpG DNA, 23, 24, 32
- Apoptosis,
 - B cell inhibition by CpG DNA, 106–108
 - immunostimulatory sequence inhibition in colitis, 376, 379, 380
- Arthritis,
 - CpG DNA induction, 354–356
 - rheumatoid arthritis,
 - adjuvant arthritis model, interferon- γ response, 368
 - Mtb DNA induction and tissue distribution, 366, 369
 - overview, 366
 - RANKL response, 368–370
 - TLR9 mediation, 370
 - DNA in joints, 365
 - immunology, 363, 364
 - models, 364
 - septic arthritis and immunostimulatory sequences, 366
 - viral DNA in joints, 354
- Asthma,
 - cytokine response, 301, 304, 307
 - DNA vaccines, 291, 292
 - epidemiology, 289, 290, 303, 304
 - hygiene hypothesis, 289, 290, 303, 304
 - immunostimulatory sequence therapy,
 - administration routes, 304
 - Amba1–immunostimulatory sequence conjugate,
 - 180–182, 185, 186, 295, 296
 - cell–cell interaction modulation, 308
 - dose-response, 304, 306, 307
 - mechanisms of action, 293, 294, 307, 308
 - ovalbumin–immunostimulatory sequence conjugate effects in mouse, 183–185
 - prospects for clinical use, 296, 309
 - signaling, 302, 303
 - Th1 response, 292–294, 302, 303, 307
 - Th2 inhibition, 293–295, 304, 308
 - Th2 response, 289, 301
 - treatment,
 - immunotherapy overview, 290, 291, 309
 - options, 302
- Autoimmune disease, *see also specific diseases*,
 - DNA antigenicity, antigenic structure, 347
 - bacterial DNA, 343, 344
 - CpG motif relationship to antigenic sequences, 344, 345
 - dogma, 341, 342
 - epitope distribution, 345–347
 - host defense implications, 347
 - immunochemical properties of antibodies, 345
 - pathogenesis, 347, 348
 - sequence contributions, 342, 343
 - systemic lupus erythematosus, 341, 342
- myocarditis, CpG DNA adjuvant effects, 392
- safety of DNA vaccines, 348, 390–392

B

Bacillus Calmette-Guerin, *see*
Mycobacterium bovis bacillus
Calmette-Guerin

B cell, *see also* Humoral immunity,
CpG DNA effects,
apoptosis inhibition, 106–108
binding, 110, 112
costimulatory molecule
expression, 105, 163, 164,
166
cytokine induction, 104, 105
endosomal maturation
requirement, 113, 114
mitogen-activated protein
kinase pathway activation,
114, 115
mitogenic activity, 103, 104
nuclear factor- κ B activation,
115, 116
TLR9 requirement, 114, 117
transcription activation, 116,
117
uptake, 112, 113
phenotype effects of CpG DNA
therapy in lymphoma, 333
phosphorothioate CpG analog,
backbone in mechanisms of
action, 108–110
effects on proliferation, 64
Toll-like receptor expression, 105,
106, 114

BCG, *see* *Mycobacterium bovis*
bacillus Calmette-Guerin

C

Cancer, *see* Lymphoma; Thymoma

CD8+ T cell,
antigen–immunostimulatory
sequence conjugate response,
177, 179, 180, 184

antigen-presenting cell cross-
presentation mechanisms,
139, 140
bystander stimulation,
interferons, 130–133
interleukin-15 control and
survival effects, 132, 133
cytotoxic T lymphocyte response
to CpG DNA,
cross-priming,
clinical applications, 144,
146
costimulatory molecules,
142–144
immunization studies, 137,
144
signals, 138, 139
TAP activity, 139–141, 144,
145
overview, 92, 96, 175, 176
gp120-immunostimulatory
sequence vaccine response,
244, 245, 247–249
memory cell development, 129,
130
mucosal immunostimulatory
sequence vaccine, splenic
cytotoxic T cell response,
193, 194, 197
plasmid vector priming influences,
222, 223
Colitis, *see* Inflammatory bowel
disease
Conjunctivitis, *see* Allergic
conjunctivitis
CpG DNA, *see also*
Immunostimulatory sequence,
adjuvant effects, *see* Dendritic
cell; Macrophage; Vaccines
applications of
immunostimulatory
sequences, 6, 7

bacterial distribution and immunostimulatory activity, 11, 13

endocytosis,
 B cells, 113, 114
 endosomal maturation, 30, 31, 44, 46
 liposomal delivery, *see* Liposomal delivery, immunostimulatory sequences
 overview, 30, 31, 44, 46

genomic frequencies, 3, 91, 175, 329, 330, 352

hexamers of immunostimulatory sequences, 3

kinetics of immune response, 255, 256

methylation, 175, 330

pathogen-associated molecular pattern criteria, 239, 240

pathogen immunoprotective activity, *see specific pathogens*

phosphorothioate oligonucleotides, *see* Phosphorothioate CpG analogs

receptor, *see* Toll-like receptor signal transduction model, 31–33

Cross-priming, *see* CD8+ T cell

D

DC, *see* Dendritic cell

Delayed-type hypersensitivity (DTH), liposome-delivered immunostimulatory sequence response, 208, 213

Dendritic cell (DC),
 antigen–immunostimulatory sequence conjugate activation mechanisms, 183–185

antigen processing effects of CpG DNA, 94, 95, 97

DNA-dependent protein kinase (DNA-PK),
 CpG DNA activation, 5, 6, 29, 30, 53, 56
 functional overview, 51, 155
 interleukin-6 and interleukin-12 induction, 52
 Rag knockout mouse response studies, 52, 53
 subunits, 29, 51
 TLR9 signaling interactions, 44, 57

DNA-PK, *see* DNA-dependent protein kinase

DNBS-induced colitis, *see* Inflammatory bowel disease

Double-stranded RNA (ds-RNA),
 innate immune response, IKK role, 49–51
 PKR role, 49–51
 prospects for study, 56
 Jun N-terminal kinase activation, 56

ds-RNA, *see* Double-stranded RNA

DSS-induced colitis, *see* Inflammatory bowel disease

DTH, *see* Delayed-type hypersensitivity

E

ERK, *see* Extracellular signal-regulated kinase

Extracellular signal-regulated kinase (ERK), *see* Mitogen-activated protein kinase

F

Francisella tularensis,
 immunoprotective activity of CpG DNA,

- clinical prospects, 260, 261
- cytokine modulation, 256, 259, 260
- duration of immunity, 257, 258
- long-term immunity induction, 258, 259
- prolongation through multiple treatments, 258
- survival effects in mice, 256, 257

G

gp120,

- immunostimulatory sequence as vaccine adjuvant,
 - CD4+ T cell-independent responses, 244, 245, 247–249
- cytotoxic T lymphocyte response, 244, 245, 247–249
- major histocompatibility complex class I-restricted responses, 244
- mouse model, 240, 241
- mucosal immune response, 243, 248
- overview of immune response, 250
- Th1 immunity and cytokine production, 241, 243, 248
- immunostimulatory sequence conjugate,
 - immune response, 179, 180, 185
- mucosal immunostimulatory sequence vaccine, 196
- potency compared with unconjugated adjuvant, 248, 249

H

HBV, *see* Hepatitis B virus

Hepatitis B virus (HBV),

- CpG DNA as vaccine adjuvant, comparison with other adjuvants,
 - mucosal antigens, 234–236
 - parenteral adjuvants, 232–234
- mice, 231
- mucosal immunostimulatory sequence vaccine, 196
- orangutans, 230, 231
- potential, 236
- rationale, 230, 236
- Th1 response, 232, 233, 236

epidemiology, 229

vaccine,

- antigens, 229
- use, 229, 230

HIV, *see* Human immunodeficiency virus

Human immunodeficiency virus (HIV), *see* gp120

Humoral immunity, *see also* B cell,

- allergic conjunctivitis
 - immunostimulatory sequence therapy, 320
- antigen–immunostimulatory sequence conjugate humoral immunity, 177, 178, 180
- liposome-delivered immunostimulatory sequences,
 - intramuscular delivery and humoral response, 205, 208, 213, 214
 - mucosal antiviral response, 208, 210
- mucosal immunostimulatory sequence vaccine, immunoglobulin A response, 190, 191, 197
- systemic lupus erythematosus, DNA antigenicity,

- antigenic structure, 347
- bacterial DNA, 343, 344
- CpG motif relationship to
 - antigenic sequences, 344, 345
- dogma, 341, 342
- epitope distribution, 345–347
- host defense implications, 347
- immunochemical properties of
 - antibodies, 345
- overview, 341, 342
- pathogenesis, 347, 348
- safety of DNA vaccines, 348
- sequence contributions, 342, 343
- Hygiene hypothesis, asthma, 289, 290, 303, 304
- I**
- IBD, *see* Inflammatory bowel disease
- IDO, *see* Indoleamine 2,3-dioxygenase
- IKK,
 - CpG DNA induction of nuclear factor- κ B, 21, 22, 50, 51
 - double-stranded RNA innate immune response role, 49–51
 - functional overview, 20
 - PKR activation, 50, 51
 - subunits, 20, 50
- IL-6, *see* Interleukin-6
- IL-10, *see* Interleukin-10
- IL-12, *see* Interleukin-12
- IL-15, *see* Interleukin-15
- Immunostimulatory sequence (ISS), *see also* CpG DNA,
 - antigen conjugates, *see* Antigen–immunostimulatory sequence conjugates
 - bacterial antigen comparison, 363, 364
 - definition, 91, 352
 - mucosal immunization, *see* Mucosal immunostimulatory sequence vaccine
 - vaccines, *see* Antigen–immunostimulatory sequence conjugates; Liposomal delivery, immunostimulatory sequences; Mucosal immunostimulatory sequence vaccine; Plasmid vectors; *Mycobacterium tuberculosis*
- Indoleamine 2,3-dioxygenase (IDO), CpG DNA induction and pathogen immunity, 270
- Inflammation, CpG DNA induction,
 - adjuvanticity mechanism comparison, 357
 - arthritis, 354–356
 - avoidance, 357, 358
 - diseases, 353, 354
 - effector mechanisms, 356, 357
 - local inflammation, 386
 - meningitis, 356, 357
 - models, 354–356
 - respiratory inflammation, 355
 - safety, 358
- Inflammatory bowel disease (IBD),
 - immunostimulatory sequence therapy,
 - DNBS-induced colitis mouse model studies, 377
 - DSS-induced colitis mouse model studies,
 - apoptosis inhibition, 376, 379, 380
 - disease activity index determination and response, 374, 375
 - induction, 374
 - inflammation reduction, 376
 - myeloperoxidase assay, 374

- human colonic cytokine
 - generation effects, 378, 380
- interleukin-10 knockout mouse
 - effects, 377–380
 - mechanisms of action, 378, 379
 - rationale, 374
- T cell response, 373
- treatment, 373, 374
- Interferons,
 - adjuvant arthritis interferon- γ response, 368
 - allergic conjunctivitis
 - immunostimulatory sequence therapy, interferon- γ response, 319, 320
 - bystander stimulation of memory T cells, 130–133
 - immunoprotective activity of CpG DNA, 256, 259, 260
- Interleukin-6 (IL-6),
 - DNA-dependent protein kinase induction, 52
 - immunoprotective activity of CpG DNA, 256, 259, 260
- Interleukin-10 (IL-10), knockout
 - mouse effects of immunostimulatory sequences, 377–380
- Interleukin-12 (IL-12),
 - CpG DNA induction in mice, 163, 165
 - DNA-dependent protein kinase induction, 52
- Interleukin-15 (IL-15), bystander stimulation control of memory T cells, 132, 133
- ISS, *see* Immunostimulatory sequence
- J**
- JNK, *see* Jun N-terminal kinase
- Jun N-terminal kinase (JNK), *see also* Mitogen-activated protein kinase,
 - CpG DNA activation, 25
 - double-stranded RNA activation, 56
- L**
- Leishmania major*, immunoprotective activity of CpG DNA, 265, 266
- Lipopolysaccharide (LPS),
 - inflammation role, 357
 - Nod proteins, cell activation role, 6, 30
 - receptor, *see* Toll-like receptor
- Liposomal delivery,
 - immunostimulatory sequences, delayed-type hypersensitivity response, 208, 213
 - encapsulation efficiency, 204, 205, 210
 - intramuscular delivery and humoral response, 205, 208, 213, 214
 - mucosal antiviral response, 208, 210
 - rationale, 203, 204
 - safety, 214
 - Th1 response, 212, 213
- Listeria monocytogenes*,
 - immunoprotective activity of CpG DNA,
 - clinical prospects, 260, 261
 - cytokine modulation, 256, 259, 260
 - duration of immunity, 257, 258
 - long-term immunity induction, 258, 259
 - prolongation through multiple treatments, 258
 - survival effects in mice, 256, 257
- LPS, *see* Lipopolysaccharide
- Lymphoma,

- CD20 as therapeutic target, 333
 - CpG DNA therapy with
 - monoclonal antibodies,
 - B cell,
 - phenotype effects, 333
 - proliferation concerns, 333, 334
 - dose-response and regimen effects, 332
 - mechanisms of action, 330, 332
 - prospects for clinical use, 334
 - rationale, 329, 330
 - survival outcomes, 330, 331
 - models, 333
- M**
- Macrophage,
 - antigen processing effects of CpG DNA, 92–94, 97
 - mitogen-activated protein kinase pathway activation by CpG DNA, 115
 - Mycobacterium avium*,
 - growth inhibition by CpG DNA, 273
 - infection, 265
 - phosphorothioate CpG analogs,
 - activation, 65–68
 - macrophage uptake, 70, 71
 - transcription-activating effects of CpG DNA, 117
 - Major histocompatibility complex (MHC),
 - CpG DNA effects on molecule synthesis, 91, 92, 94, 95, 97
 - memory cell development role, 130
 - MAPK, *see* Mitogen-activated protein kinase
 - Memory T cell, *see* CD8+ T cell
 - Meningitis, CpG DNA induction, 356
 - MHC, *see* Major histocompatibility complex
 - Mitochondrial DNA, bacterial DNA similarities, 353, 355
 - Mitogen-activated protein kinase (MAPK), *see also* Jun N-terminal kinase,
 - cascade overview, 23
 - CpG DNA activation of
 - extracellular signal-regulated kinase pathway,
 - AP-1 activation, 23, 24
 - B cells, 114, 115
 - cell-type specificity, 24, 25
 - immune cell effector function regulation, 25, 26
 - macrophages, 115
 - cytokine regulation by p38, 25, 26
 - phosphorothioate CpG analog activation, 68–70
 - types, 23
- MS, *see* Multiple sclerosis
- Mucosal immunostimulatory
 - sequence vaccine,
 - adjuvant applications,
 - gp120, 196
 - hepatitis B surface antigen, 196
 - ovalbumin, 195
 - polysaccharide antigens, 196, 197
 - cytokine response, 191–193
 - immunoglobulin A response, 190, 191, 197
 - prepriming, 194, 195, 198
 - rationale, 189, 198, 199
 - splenic cytotoxic T cell response, 193, 194, 197
 - Th1 response, 191, 197
- Multiple sclerosis (MS), CpG DNA exacerbation, 392
- MY-1, *see* *Mycobacterium bovis* bacillus Calmette-Guerin
- Mycobacterium avium*,
 - immunoprotective activity of CpG DNA,

- chemotherapy adjunct efficacy, 270, 272, 273
- growth inhibition studies,
 - in vitro, 266, 273
 - in vivo, 266, 269
 - mechanisms of action,
 - indoleamine 2,3-dioxygenase induction, 270
 - negative finding studies, 269, 270
- macrophage,
 - growth inhibition, 273
 - infection, 265
- opportunistic infection, 265
- Mycobacterium bovis* bacillus Calmette-Guerin (BCG),
 - adjuvant arthritis, Mtb DNA induction and tissue distribution, 366, 369
- anti-tumor activity of DNA, 3, 9–11, 81
- bladder cancer treatment, 329
- MY-1
 - composition, 10
 - DNA sequences, 10, 11
 - historical perspective, 220
 - natural killer cell activation,
 - antitumor activity
 - correlation, 83
 - base length effects, 83, 85
 - human peripheral blood lymphocyte activation, 86–88
 - mouse spleen cell lipofection effects, 85, 86
 - overview, 81, 82
 - sequence specificity, 82, 83
- Myd88
 - independent cell activation by TLR ligands, 6
 - Toll-like receptor signaling complex, 6, 18, 19, 27, 39, 40
- N**
- Natural killer (NK) cell,
 - CpG DNA activation mechanisms, 155, 163
- MY-1 activation,
 - antitumor activity correlation, 83
 - base length effects, 83, 85
 - human peripheral blood lymphocyte activation, 86–88
 - mouse spleen cell lipofection effects, 85, 86
 - overview, 81, 82
 - sequence specificity, 82, 83
 - phosphorothioate CpG analog activation, 64, 65
- NF- κ B, *see* Nuclear factor- κ B
- Nitric oxide (NO), CpG DNA-induced inflammation role, 356
- NK cell, *see* Natural killer cell
- NO, *see* Nitric oxide
- Nod1
 - cell distribution, 30
 - domains, 30
 - lipopolysaccharide-induced cell activation role, 6, 30
- Nod2
 - cell distribution, 30
 - domains, 30
 - lipopolysaccharide-induced cell activation role, 6, 30
- Nuclear factor- κ B (NF- κ B),
 - CpG DNA activation,
 - B cells, 115, 116
 - effector function modulation, 22, 23
 - inhibitory protein kinase complex modulation, 21, 22
 - inhibitory proteins, *see* IKK
 - kinase induction, 21
 - subunits, 20

O

- Ovalbumin–immunostimulatory sequence conjugate, effects on mouse asthma model, 183–185
- tumor vaccine, 184

P

- p38, *see* Mitogen-activated protein kinase
- PAMPs, *see* Pathogen-associated molecular patterns
- Pathogen-associated molecular patterns (PAMPs), *see* CpG DNA; Double-stranded RNA; Lipopolysaccharide
- Phosphorothioate CpG analogs, applications, 63, 73, 74
- backbone in mechanisms of B cell action, 108–110
- inhibitory activity at high concentrations, 71, 72
- macrophage uptake, 70, 71
- pharmacokinetics, 386
- potency studies,
 - B cell proliferation, 64
 - macrophage activation, 65–68
 - natural killer cell activation, 64, 65
- protein interactions with backbone, 72, 73
- rodent versus primate safety studies, 388
- signaling events, 68–70
- stability, 63, 352
- toxicity and safety, 385–388, 393
- PKR, double-stranded RNA innate immune response role, 49–51
- Plasmid vectors, design considerations, 221

- historical perspective, 220
- immunostimulatory sequence consensus motif, 220, 22
- T cell priming influences, 222, 223
- Th1 response, 219, 222
- TLR9 mediation, 222, 223

R

- RANKL, adjuvant arthritis response, 368–370
- Rheumatoid arthritis, *see* Arthritis

S

- Selectins, CpG DNA-induced inflammation role, 356, 357
- SLE, *see* Systemic lupus erythematosus
- Systemic lupus erythematosus (SLE), chloroquine therapy, 390, 391
- CpG DNA,
 - levels in circulation, 354, 391
 - mouse model studies, 390, 391
 - peripheral blood mononuclear cell response, 391, 392
- DNA antigenicity,
 - antigenic structure, 347
 - bacterial DNA, 343, 344
- CpG motif relationship to antigenic sequences, 344, 345
- dogma, 341, 342
- epitope distribution, 345–347
- host defense implications, 347
- immunochemical properties of antibodies, 345
- overview, 341, 342
- pathogenesis, 347, 348
- safety of DNA vaccines, 348
- sequence contributions, 342, 343

T

TAP, CD8+ T cell cross-priming activity, 139–141, 144, 145

T cell,

cytotoxic T lymphocyte, *see* CD8+ T cell

gp120-immunostimulatory sequence vaccine,

CD4+ T cell-independent responses, 244, 245, 247–249

cytotoxic T lymphocyte response, 244, 245, 247–249

Th1 immunity and cytokine production, 241, 243, 248

human peripheral blood lymphocyte activation by MY-1, 86–88

inflammatory bowel disease response, 373

plasmid vector priming influences, 222, 223

T helper cell response, *see* Th1; Th2

treatment, 373, 374

Th1,

antigen–immunostimulatory sequence conjugate response, 177, 180–182

asthma immunostimulatory sequence therapy response, 292–294, 302, 303, 307

bacterial product toxicity synergism following polarization, 387

CpG DNA induction, allergy protection in animals, 156, 157, 171

antigen-presenting cell mediation, 165

clinical applications, 172

gp120-immunostimulatory sequence vaccine immunity and cytokine production, 241, 243, 248

liposome-delivered immunostimulatory sequences, 212, 213

mucosal immunostimulatory sequence vaccine response, 191, 197

overview, 92, 95, 137

prepriming of immune response and duration, 165–168, 170, 171

TLR9 requirement, 154

tumor effects, 353

cytokine profile and response, 153, 154, 171, 172

duration of CpG DNA-induced polarization, 389, 390

Mycobacterium tuberculosis response, 170

plasmid vector response, 219, 222

Th2,

allergen–immunostimulatory sequence conjugates and Th2 downregulation, 180–182, 185, 186

allergy response, 156, 157

asthma,

immunostimulatory sequence therapy inhibition, 293–295, 304, 308

response, 289, 301

CpG DNA prepriming and inhibition of response, 168, 169, 171

- cytokine profile and response, 153, 154
- diseases, *see* Allergy; Asthma
- DNA vaccine induction, 156
- Thymoma, ovalbumin–
 - immunostimulatory sequence
 - conjugate as tumor vaccine, 184
- TLR, *see* Toll-like receptor
- Toll-like receptor (TLR),
 - B cell expression, 105, 106
 - conservation between species, 4, 39
 - cytokine activation, 5
 - signal transduction, *see also*
 - Mitogen-activated protein kinase; Nuclear factor- κ B,
 - evidence for CpG DNA
 - activation, 26, 27
 - oligomerization, 18
 - prospects for study, 33
 - signaling complex, 4–6, 18, 19, 27
- TLR2
 - function, 39
 - ligands, 18
- TLR4
 - function, 39
 - ligands, 18
 - lipopolysaccharide binding, 105
 - signaling, 105
- TLR9
 - B cells, 114, 117
 - discovery, 40
 - endocytosis, 30, 31, 44, 46
 - immunostimulatory sequence
 - signaling, 5, 6, 27–29
 - knockout mouse,
 - cellular response to CpG DNA, 40, 41
 - guanosine-rich
 - oligodeoxynucleotide responses, 43, 44
 - in vivo response, 42, 43
 - structure, 27
 - types, 4
- TRAF6, Toll-like receptor signaling complex, 6, 18, 19, 27
- V
- Vaccines, *see* Antigen–
 - immunostimulatory sequence
 - conjugates; Liposomal delivery,
 - immunostimulatory sequences;
 - Mucosal immunostimulatory sequence vaccine; Plasmid vectors; *Mycobacterium tuberculosis*

Microbial DNA and Host Immunity

Edited by

Eyal Raz, MD

University of California, San Diego, La Jolla, CA

The immunostimulatory prospects of bacterial DNA have attracted the interest and attention of scientists and physicians and become a major focus in immunobiology and biomedicine. These activities are the product of immunostimulatory DNA sequences (ISS, also known as GpG motifs), which are rare in the mammalian genome. ISS were shown to enhance immunological responses and were used to confer protection to a wide variety of tumors, allergic inflammation, and infections. In *Microbial DNA and Host Immunity*, leading researchers review the activation of the mammalian immune system by bacterial DNA and consider the applications of ISS in clinical medicine. The authors survey the latest findings concerning the receptor-recognition and signaling pathways triggered by ISS, the process of cell activation, and potential vaccination strategies using ISS. Specific pharmaceutical applications discussed include infectious disease (Hepatitis B, HIV, and mycobacterial infections), allergy (asthma and conjunctivitis), cancer (lymphoma), and inflammation and autoimmunity (arthritis and colitis).

Up-to-date and informative, *Microbial DNA and Host Immunity* illuminates the immunobiology of bacterial DNA and its promise of powerful new vaccines to provide protective immunity against infections, tumors, and chronic disease.

FEATURES

- Cutting-edge reviews of immune activation by bacterial DNA
- Vaccine applications in infectious disease, allergy, cancer, inflammation, and autoimmunity
- Vaccination strategies for utilizing immunostimulatory sequences in bacterial DNA
- New light on TLR signaling pathway and its microbial ligands

CONTENTS

Part I: Introduction. Immunostimulatory DNA: *An Overview*. Historical Perspectives. **Part II: Receptors and Signaling.** Signal Transduction Pathways Activated by CpG-DNA. A Novel Toll-Like Receptor that Recognizes Bacterial DNA. Activation of Innate Immunity by Microbial Nucleic Acids. Phosphorothioate Backbone Modification Changes the Pattern of Responses to CpG. **Part III: Cell Activation.** Activation of NK Cell by Immunostimulatory Oligo-DNA in Mouse and Human. Regulation of Antigen Presenting Cell Function by CpG-DNA. Activation of B Cells by CpG Motifs in Bacterial DNA. IFN-Dependent Pathways for Stimulation of Memory CD8+ Cells. Cross Priming of CD8+ T Cells by Immunostimulatory Sequence DNA. **Part IV: Vaccination Strategies.** The Th1 Adjuvant Effect of Immunostimulatory DNA Sequences. Immunostimulatory DNA Pre-Priming for the Induction of Th1 and Prevention of Th2 Biased Immune Responses. Protein-Immunostimulatory DNA-Conjugate: *A Novel Immunogen*. Immunostimulatory DNA Sequence-Based Mucosal Vaccines. Enhancement of the Immunoadjuvant Activity of Immunostimulatory DNA Sequence by Liposomal Delivery. Immunostimulatory Sequences in Plasmid Vectors.

Part V: Applications—Infectious Disease. Comparison of CpG DNA with Other Adjuvants for Vaccination Against Hepatitis B. Immunostimulatory DNA-Based Immunization: *Hope for an HIV Vaccine?* Immunoprotective Activity of CpG Oligonucleotides. Protective Immunity of Immunostimulatory Sequences Against Mycobacterial Infection. **Part VI: Applications—Allergy.** DNA-Based Immunotherapeutics for Allergic Disease. Immunostimulatory DNA for Allergic Asthma. CpG Oligodeoxynucleotides in Asthma. Modulation of Allergic Conjunctivitis by Immunostimulatory DNA Sequence Oligonucleotides. **Part VII: Applications—Cancer.** CpG Oligodeoxynucleotides and Monoclonal Antibody Therapy of Lymphoma. **Part VIII: Inflammation and Autoimmunity.** The Antigenicity of Bacterial DNA. Inflammatory Properties of Immunostimulatory DNA Sequences. The Role of Immunostimulatory DNA Sequences in Arthritis. Effects of Immunostimulatory DNA Oligonucleotides on Experimental Colitis. **Part IX: Safety Considerations.** CpG ODN: *Safety Considerations*. Index.

ISBN 1-58829-022-0



MICROBIAL DNA AND HOST IMMUNITY
ISBN: 1-58829-022-0

humanapress.com