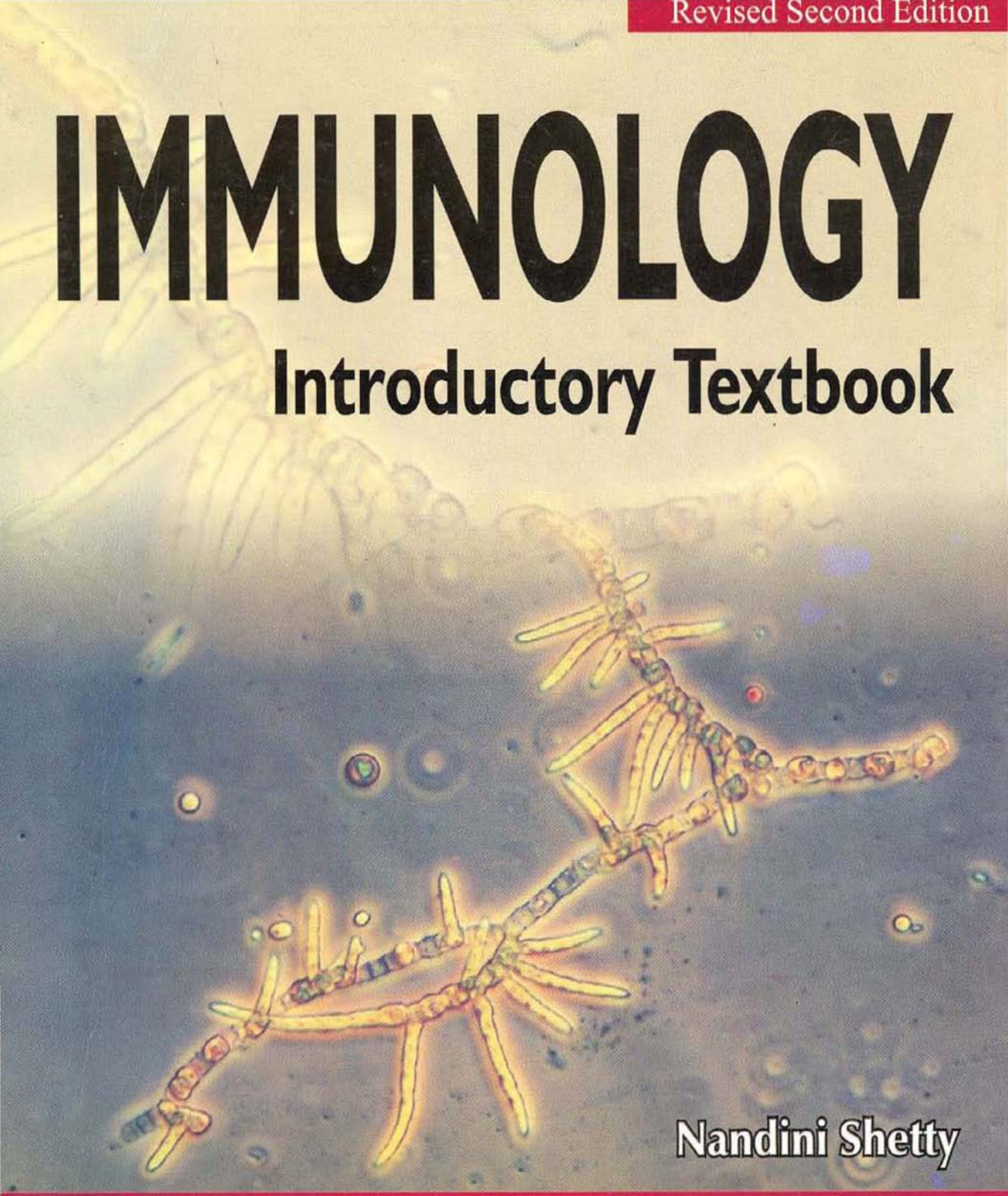
Revised Second Edition

IMMUNOLOGY

Introductory Textbook



Nandini Shetty

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Introductory Textbook

Revised Second Edition

Nandini Shetty
Consultant Microbiologist
Health Protection Agency Collaborating Centre
University College London Hospitals
London, UK

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PREFACE TO THE SECOND EDITION

As textbooks and journals of immunology grow in size and number and the explosion of information floods the libraries, students often find themselves overwhelmed by the volume of reading required to understand basic concepts. Assimilating a fast changing, ever expanding subject like immunology can be a daunting process. As a teacher of immunology and clinical microbiology for undergraduate medical students, I have tried to present the basic tenets of the subject of immunology in a simple and understandable manner, very much like the many lectures I have given to students over the years. I have tried to evolve a story so that students may be inspired to read on and experience the wonder of scientific discovery. To this end I owe my own enjoyment of the subject to the many excellent articles in 'Scientific American Medicine', to the pictorial presentation of immunological concepts in Ivan Roitt's excellent textbooks of Immunology and to 'Basic and Clinical Immunology' of the Lange Medical Publication series. I gratefully acknowledge these sources for the many ideas and figures that I have adapted for this book. The second edition has been carefully updated, with some chapters being rewritten incorporating several new illustrations, in keeping with scientific advances. A new chapter dedicated to the immunology of HIV has been added in recognition of the devastating pandemic that has re-defined global health. I hope the book will be of use to students of medicine, microbiology, nursing and for any one else in the life sciences who feels the need to explore this fascinating area of science.

London **Nandini Shetty**

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PREFACE TO THE FIRST EDITION

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London **Nandini Shetty**

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LIST OF ABBREVIATIONS

Chapter 1

BCG	Bacille Calmette Guerin
C region	Constant region
Fab	Fraction antigen binding
Fc	Fraction crystallizable
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
T cell	Thymus derived cell or thymocyte
V region	Variable region

Chapter 2

CRP	C-reactive protein
IL-1	
IL-2	
IL-3	
IL-4	Interleukins-1 to 7
IL-5	
IL-6	
IL-7	
NADPH	Nicotinamide adenosine dinucleotide phosphate hydrogen

Chapter 3

CD	Cluster of differentiation
GALT	Gut associated lymphoid tissue
MALT	Mucosal associated lymphoid tissue
MBP	Major basic protein
MPS	Mononuclear phagocyte system
NK	Natural killer
PMN	Polymorphonuclear neutrophil

Chapter 4

DNP	Dinitrophenyl

Chapter 5

CDR	Complementarity determining regions
C_H	Constant heavy
C_L	Constant light
H chain	Heavy chain
L chanin	Light chain
V_H	Variable heavy
V_L	Variable light

Chapter 6

D region	Diversity
IVS	Intervening sequences
J region	Joining region

Chapter 7

P	Properdin
SLE	Systemic lupus erythematosus
SRS-A	Slow reacting substance-A

Chapter 8

CEA	Carcinoembryonic antigen
CIE	Counter immunoelectrophoresis
ELISA	Enzyme linked immunosorbent assay
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
IEP	Immunoelectrophoresis
IHA/PHA	Indirect/Passive haemagglutination
RAST	Radio allergosorbent test
RIA	Radio immunoassay
RIST	Radio immunosorbent test
RPHA	Reverse passive haemagglutination
SDS-Page	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis

Chapter 9

HAT	Hypoxanthine aminopterin thymidine
HPRT	Hypoxanthine phosphoribosyl transferase
PEG	Polye thylene glycol

Chapter 10

GBM	Glomerular basement membrane
HTC	Homozygous typing cells
Ir genes	Immune response genes
Is genes	Immune suppressor genes

MLR	Mixed lymphocyte reaction
PLT	Primed lymphocyte typing

Chapter 11

TCR	T cell receptor

Chapter 12

APC	Antigen presenting cell
BCDF	B cell differentiation factor
BCGF	B cell growth factor
ICAM	Intercellular adhesion molecule
IFN	Interferon
IP3	Inositol triphosphate
LFA	Lymphocyte function associated antigen
Tc	T cytotoxic
Th	T helper

Chapter 13

ADCC	Antibody dependent cellular cytotoxicity
BAF	B cell activating factor
LAF	Lymphokine activating factor
LPS	Lipopolysaccharide
MAF	Macrophage activating factor
MDP	Muramyl dipeptide
MIF	Migration inhibitory factor
Multi-CSF	Multi-Colony stimulating factor

Chapter 14

CMI	Cell mediated immunity
EBV	Epstein Barr virus
ECF	Eosinophil chemotactic factor
ESP	Eosinphil stimulatory promoter
FACS	Fluorescence activated cell sorter
GM-CSF	Granulocyte-monocyte colony stimulating factor
HTLV 1	Human T cell leukemia virus 1
HTLV 2	Human T cell leukemia virus 2
LIF	Lymphocyte inhibitory factor
LT	Lymphotoxin
OAF	Osteoclast activating factor
PHA	Phyto haemagglutinin
PPD	Purified protein derivative
PWM	Pokeweed mitogen
TNF	Tumour necrosis factor

Chapter 15

ECF-A	Eosinophil chemotactic factor-anaphylaxis
NCF-A	Neutrophil chemotactic factor-anaphylaxis
PK	Prausnitz küstner

Chapter 16

Ab 1, 2, 3	Antibody 1, 2, 3
BSA	Bovine serum albumin
Id 1, 2, 3	Idiotype 1, 2, 3
LATS	Long acting thyroid stimulator
NZB	New Zealand black
NZW	New Zealand white

Chapter 17

LAK	Lymphokine activated killer

Chapter 18

GVH	Graft versus host
SCID	Severe combined immunodeficiency

Chapter 19

DNFB	Dinitroflurobenzene
FeLV	Feline leukemia virus
MCA	Methyl cholanthrene

Chapter 21

HIG	Human immunoglobulin
VZIG	Varicella zoster immune globulin

Chapter 22

ADA	Adenosine deaminase
AIDS	Acquired immunodeficiency sundrome
CVID	Combined variable immunodeficiency

CONTENTS

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CHAPTER – 1

MILESTONES IN IMMUNOLOGY

With the evolution of the germ theory of disease, the study of the mechanisms of immunity and the possible conquest of infectious diseases began almost simultaneously. Interest in immunology grew out of the everyday evidence that those individuals who survived: pocked and disfigured from the dread disease, small pox never did contract the infection again. As early as 1773, Voltaire reported on an ancient Chinese custom where dried and powdered small pox scabs were inhaled, much like snuff, in at attempt at preventing the disease. Although it would take several years before bacteria and viruses were fully characterized, theorists were already elucidating the mechanisms of host defence against infectious diseases and their possible prevention.

The **phagocytic theory** was the first to be developed in the 1880s a bold and imaginative concept — the fruit of **Elie Metchnikoff's** deep knowledge of biology. Metchnikoff hypothesized that the basis of inflammation was the cellular reaction and that vascular and nervous reactions were only of secondary importance. He postulated further that these migrating cells which were able to move in order to meet an enemy were the major guardians of health against bacterial infections. It was around this time that **Louis Pasteur** reported on the treatment of his first two patients with rabies using early vaccines. In 1888 Metchnikoff left Russia to continue his work on phagocytosis in Pasteur's Institute in Paris. In 1908 he shared the Nobel Prize with Paul Ehrlich for his early contributions to the understanding of inflammation.

After Metchnikoff died in 1916 other workers who had long cherished the theory that **soluble substances** in blood were also bactericidal, reported their observations. **Behring**, **Nuttall** and **Nissen** contributed substantially to the concept of soluble or **humoral factors** but found that these factors were not always bactericidal. Finally in 1903, **Almroth Wright** with his disciple **Stewart Douglas** observed that a humoral component which they designated **opsonin**, could render bacteria susceptible to phagocytosis. They thus forged a link between the two major theories of immunity. Since the time humoral and cellular components were shown to be interwoven in an intricate pattern, the complex chemicals associated with immunity have been the continuing subject of years of research.

Mere empirical methods of producing or increasing immunity were not sufficient, the whole **basis of immunity** needed questioning. Behring found that certain diseases were the expression of the action of **toxins** which could be neutralized by **antitoxins**. And it could not be ignored that the blood of animals contained certain preformed bactericidal agents. **Emil von Behring** and **Kitasato** in 1890 inoculated animals with toxins of diphtheria and tetanus, to produce neutralizing antitoxin serum. They introduced **passive immunization** into modern medicine and for this, von Behring was awarded the French legion of honour and the Nobel Prize in 1901. Behring maintained cordial ties with Pasteur - one of his great heroes and with Metchnikoff he established a continuing friendship that began in 1888. This friendship was unique because the two men were at opposite poles in explaining basic aspects of immunity -yet enjoying the stimulus of intellectual debate.

Though eminently successful in passive immunization against diphtheria and tetanus, von Behring failed abysmally when he tried to extend these principles to passive immunization against tuberculosis. At this time **Robert Koch** too had to taste failure in his attempts at providing a successful vaccine against tuberculosis. The prevention of tuberculosis was not to be found by the Germans. It was in France that **Albert Calmette** and **Camille Guerin** developed an effective **vaccine for tuberculosis** by the methods of attenuation so dear to Pasteur and disdained by Koch.

To establish a live but attenuated strain of *Mycobacterium tuberculosis* they had to continually transfer or sub culture the organism till virulence was lost. This odyssey took them thirteen years and 230 transfers later, on Jan. 5, 1921 it was completely avirulent even at high doses for all animal species. The **Bacille Calmette Guerin** (BCG) was thus born - it did not induce the formation of tubercles by intravenous, intraperitoneal or subcutaneous inoculation or even by ingestion, but formed an effective prophylactic against tuberculosis.

Pfeiffer in 1894-95, discovered the phenomenon of in–vivo cytolysis of *Vibrio cholerae* when the organism and immune serum interacted intraperitoneally in the guinea pig. From these early experiments the nature and function of **complement mediated cytolysis** were elucidated by **Pfeiffer** and later by **Buchner** and **Bordet**. Jules Bordet was awarded the Nobel Prize in 1920 for his pioneering work on complement, for his discovery of the whooping cough agent and for his enunciation of the diverse aspects of antigen – antibody reactions and the blood coagulation system. Around this time **Gruber** and **Durham** described the diagnostic value of the **agglutination reaction**, when agar cultures were mixed with their corresponding immune sera. The same year **Ferdinand Widal** the Algerian born son of a French army surgeon described the agglutination reaction for typhoid fever—a test which is still widely used and bears his name.

In 1667, **Jean Baptiste Denis**, physician to Louis the XIV, performed what is considered to be the first **transfusion of blood** in man. The experiment was not well received by his fellow-physicians who were more accustomed to blood-letting than reversing the flow. Till the early 1900s the practice of transfusion of blood from man to man remained a risky, unpredictable business, when at the turn of the century, **Carl Landsteiner** discovered the blood groups. Landsteiner received the Nobel Prize in 1930 for elucidating the blood groups and for his work on the Rh factor.

As serotherapy developed, it soon became evident that antigen- antibody reactions in vivo could have some harmful effects and even produce death. **Anaphylaxis**, the most dramatic manifestation of hypersensitivity was first described by **P.Portier** and **Charles Richet** in 1902. In 1913 Charles Richet received the Nobel Prize in recognition of his work on anaphylaxis.

Two of the most vital immunological hypotheses made in the 1800s went undetected for several decades. They were rediscovered only in the 1940s and form a basis of the study of immunology today. Elie Metchnikoff suggested that phagocytes were the prime detectors of foreign material— we trace the origins of **cellular immunity** to this hypothesis. **Paul Ehrlich** proposed the pre–existence of receptors (which he called toxophores) on the living cell, that reacted with toxins. Excess receptors were liberated into the circulation as antibodies–the molecular basis of **humoral immunity**, as we know it today, is remarkably close to this novel but original concept. Thus, the foundations of modern immunology, as we recognize it today, were laid by several European scientists over the last hundred or more years.

The twentieth century saw phenomenal progress in the understanding of immunological concepts. How antigens direct the various immunological processes was the chief point of contention among early theorists who had to choose between the instructive and the selective

theories. In the past 30 years the biological basis of the immune response was, in part, clarified when the theory of **clonal selection of antibody formation** found acceptance. This theory proposed, in essence, by Paul Ehrlich almost a 100 years ago fell out of favour with the introduction of the template theory. The first comprehensive attack against the template theory was launched in 1955 by **Niels Kaj Jerne** who recalled certain criticisms made by **Macfarlane Burnet** against the template theory. It, however, remained for Burnet to draw together the new conceptualization and in 1957, he asserted that "each cell and its clones can produce just one kind of receptor" to explain the specificity of the immune response and the exponential rise in antibody production following contact with antigen. The secondary response is more powerful because antigenic memory leads to rapid clonal expansion during subsequent contact with the same antigen. We know today that during this second exposure the binding ability of antibody improves and we also know this to be the result of affinity maturation and somatic hypermutation. The clonal selection theory also explained tolerance as the deletion or suppression of an entire clone of cells which could occur before or soon after birth; or even much later in some instances. Macfarlane Burnet was awarded the Nobel Prize in 1960 which he shared with **Peter Medawar**. In 1984 Niels Jerne was also awarded the Nobel Prize for his theoretical contributions to immunology, the most fundamental of which was his role in developing he concept of clonality and for his description of the idiotype network in the regulation of immune responses.

The **elucidation of antibody structure** is credited to **Rodney R. Porter** and **Gerald M. Edelman** who shared the Nobel Prize for their discovery in 1972. In 1959 Edelman showed that the immunoglobulin molecule had 4 polypeptide chains: 2 of each kind and that each could be separated by chemical means. He called them light and heavy chains because of their size. At the same time Porter showed that the molecule could be cut into 3 different pieces (Fab x 2; Fc x 1) by enzymes that cleave polypeptides. In 1970 Edelman showed that chemical differences responsible for the specificity of antigen binding were embodied in the amino acid sequences of their variable regions at the upper, outer arms of the antibody molecule. All subsequent work has confirmed his conclusions with some elaborations. In 1970 **T.T. Wu** and **E.A Kabat** demonstrated the presence of hyper variable regions.

Dreyer and **Bennet** in 1965, suggested that the germ line contains many variable (V) region genes and one constant (C) region gene, which combine to yield an immunoglobulin molecule. As a cell matures, it selects one V gene out of many and combines it with the one C region gene. The theory was attractive; yet it required that the cell have some means of rearranging genes in somatic cells. Evidence that immunoglobulin genes do undergo **somatic recombination**, but in more complicated ways than Dreyer and Bennet suggested, was found in 1970 by **Hozumi** and **Tonegawa** then at Basel, Switzerland. They demonstrated that V and C genes were far apart in embryonic cells but much closer to each other in plasma cells. They also delineated the mechanism of shuffling of the many gene segments. Tonegawa was awarded the Nobel Prize in 1987 for elucidating the mechanism by which the immune system generates an almost limitless variety of antibodies. Somatic hypermutation, as another major source of the tremendous diversity of antibody specificity generated, was demonstrated by **Weigert** and **Cohn** in 1970.

The production of **monoclonal antibodies** by somatic cell hybridization of antibody forming cells and continuously replicating cell lines was called the technique of hybridoma formation. This technique described by **Georges Kohler** and **Cesar Milstein** in 1975, has enabled immunologists to prepare virtually unlimited quantities of antibodies that are chemically, physically and immunologically completely homogenous. For this invaluable contribution to immunology they were awarded the Nobel Prize in 1984.

Immunobiology of **transplantation** and **tolerance** was an active field of research in the 1940s and 50s. In 1953, **Medawar**, **Brent** and **Billingham** performed a series of dazzling experiments to explain the mechanisms that enable the host to recognize and destroy foreign cells. Medawar showed conclusively that such a mechanism was immunological. He suggested that specific individuality markers were associated with every cell of an organism and that an animal can be immunized against foreign grafts by first injecting it with cells derived from immunocompetent tissues of the donor. Earlier **Ray Owen** had shown, in his classic experiment with dizygotic twin calves, that dissimilar blood group antigens of one calf were tolerated by the other twin calf. Drawing from this experiment Medawar demonstrated that an inbred strain of mice – strain A could be made to tolerate skin grafts from another inbred strain – strain B by injecting the strain A animal soon after birth with strain B spleen cells. With this experiment he proved that resistance (or the lack of it) to foreign tissue is an immunological phenomenon. Peter Medawar was awarded the Nobel Prize (with Burnet) in 1960.

Existence of markers of biological individuality – what we now know as the **histocompatibility antigens** was first suggested by **Gorer** in 1937, who demonstrated that loci determining blood group antigens and those controlling tumour rejection in mice were distinct. With **Snell** in 1948 he showed that there were multiple alleles in the mouse H-2 locus and subsequently that this locus was genetically complex. The H-2 locus was shown to control the phenotype expression of certain cell surface markers in mice. In 1950 **Dausset** identified the HLA (Human Leukocyte Antigen) locus or MHC (major histocompatibility complex) as it is sometimes called. **Benacerraf** showed that genes of the HLA determining loci may control immune responses. **Snell**, **Dausset** and **Benacerraf** were awarded the Nobel Prize in 1980. Elucidation of the structures of some HLA molecules by **Bjorkman** and **Wiley** in 1987, using X-ray crystallography, revealed that the molecules of the major histocompatibility complex (MHC) bind to antigenic peptides and present these peptides to the T cell receptor.

First glimpsed in experiments by **Allison**, and **Kappler** & **Reinherz**, the **T cell receptor** was studied and characterized by **Tak W.Mak** and **Mark M. Davis** in 1984. These workers cloned and sequenced a gene expressed and rearranged in T cells but not in B cells. Their analysis showed that many of the T cell receptor sequences were homologous to those of immunoglobulin genes. **Peter Doherty** with **Rolf M. Zinkernagel,** found that T cells from mice infected with a meningitis virus destroyed virus-infected cells only from the same strain of mice, and they showed that T cells must recognize two signals on an infected cell—one from the virus and one from the cell's own antigens—to destroy it. For this new understanding of cellular immune mechanisms they shared the Nobel Prize in 1996.

Immunology continues to fascinate and frustrate researchers the world over. A better understanding of tumour immunology, autoimmunity and responses to immunization could develop into strategies for protection against many crippling diseases. Eventually, what has evolved as a precise and powerful tool created by nature to ensure the continued survival of the species could perhaps be manipulated to yield a better quality of life.

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CHAPTER – 2

INNATE IMMUNITY

Taliaferro said, "The host is an island invaded by strangers with different needs, different food requirements, different localities in which to raise their progeny". There are a formidable range of infectious agents that can use the human body as a sanctuary to raise their offspring. The immune system faces the task of providing a defence mechanism to establish a state that is known as immunity to infection.

The contemporary definition of immunity is therefore: "All those physiological mechanisms that endow the animal with the capacity to recognize materials as foreign to itself and to neutralize, eliminate or metabolize them with or without injury to its own tissues".

Immunological responses serve three broad functions:

- Defence against micro organisms
- Homeostasis; removal of damaged or effete cells
- Surveillance; recognition and destruction of mutant cells.

These responses have been widely classified as **non–specific** and **specific**. Among the non-specific defence mechanisms there are those that form a set of ill understood and perhaps grossly under-emphasized **constitutional** factors that make one species innately susceptible and another resistant to certain infections.

These **constitutional factors** can best be listed as–

(*a*) Genetic: between species, for example:

- *Mycobacterium leprae* seems to infect humans and armadillos only.
- *Bacillus anthracis* is an infection of humans though not of chickens.
- Gonorrhoea is a disease of man and chimpanzees and not of any other species. Some species seem to be able to harbour organisms within their body tissues while the same organism may cause another species to succumb to such an infection. Within man, there are certain well known **racial differences** in disease susceptibility:
  - dark skinned individuals have an increased susceptibility to coccidioidomycosis.
  - certain dark skinned people lack the red cell Duffy coat and are not susceptible to vivax malaria.

Genetic control of disease has been shown to be strongly associated with the major histocompatibility complex.

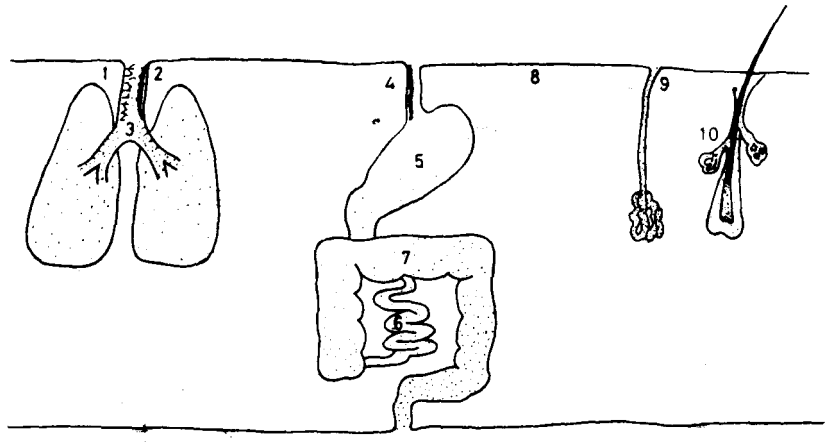
(*b*) Age: The very young are more susceptible to many infections, in particular, *Escherichia coli* meningitis; this may be because bactericidal IgM does not cross the placenta.

At the other end of the spectrum rickettsial infections and certain viral infections of children are more severe with age.

(*c*) Metabolic: Hypoadrenal and hypothyroid states decrease resistance to infection. In diseases such as diabetes mellitus where altered metabolism causes increase in blood glucose, decrease in pH and a reduced influx of phagocytes - infection can be a severe complication. Steroid hormones are known to affect many modalities of the immune response.

(*d*) There is a growing body of evidence that supports the hypothesis that immune processes can be influenced by **neuroendocrine factors**. Immunologic cells have receptors for a whole range of hormones. Corticosteroids, androgens, oestrogens and progesterone depress immune responses, whereas growth hormone, insulin and thyroxine do the opposite. Immunologic organs are innervated by autonomic and primary sensorial neurons; hormone secretion is balanced by neural control. It therefore seems reasonable to say that immune responses are finely tuned by neuro endocrine circuits. At a more physiologic level, stress and circadian rhythms modify the functioning of the immune system.

(*e*) Environment: Poor living conditions, overcrowding and undernutrition also increase susceptibility to infection.



1. Cilia
2. Mucus
3. Cough reflex
4. Mucus
5. Acid in gastric juice
6. Intestinal enzymes
7. Colonic flora
8. Intact skin
9. Sweat
10. Sebum

Figure 2.1. Natural barriers to infectious agents.

Natural barriers to infectious agents are simple yet effective means of innate defence (Figure 2.1). A major form of defence in this context is the **intact skin** which is impermeable to most infectious agents. **Sweat** and **sebaceous glands** are potential points of entry for the infectious agent. However, most bacteria fail to enter due to the low pH and direct inhibitory effects of **lysozymes**, **lactic acid** and other **fatty acids** of sweat and sebaceous secretions. An exception is *Staphylococcus aureus* which commonly infects the hair follicle and glands.

Mucus secretions of the tracts that connect internal organs to external surfaces form an important form of defence. They entrap and immobilize bacteria and hence, prevent adherence and colonization of epithelial surfaces. **Hairs at the external nares**, the **cough reflex** and the **ciliated mucus membrane** of the respiratory tract help drive entrapped organisms upwards and outwards. Other mechanical factors which help protect these tracts are the **washing**

action of tears, saliva and urine. Many secretions contain bactericidal components such as **acid in gastric juice**; **lysozyme** in tears, nasal secretions and saliva; **proteolytic enzymes** in intestinal secretions; **spermine and zinc in semen** and **lactoperoxidase in breast milk.**

An important mechanism of defence is associated with **normal bacterial flora** of the body. Normal flora can suppress the growth of many potentially pathogenic bacteria and fungi by competition for essential nutrients or by production of inhibitory substances such as colicins or acid. Pathogenic invasion of the vaginal flora is inhibited by lactic acid produced by the commensal vaginal flora. When normal human flora is destroyed by broad spectrum antibiotics, pathogens such as *Candida* spp. and *Clostridium difficile* cause opportunistic infections.

If microorganisms do penetrate the body, two main defensive operations come into play – **phagocytosis** and the bactericidal effect of **soluble chemical factors** (molecules such as complement proteins, acute phase proteins, and cytokines). Other cells may also be involved: cells that release inflammatory mediators (basophils, mast cells, and eosinophils) and natural killer cells (NK cells).

Phagocytosis

The engulfment and digestion of infectious agents is assigned to two major cell populations : the polymorphonuclear neutrophil (PMN) and the macrophage (M). Phagocytosis is a multiphasic act (Figure 2.2), requiring recognition, movement of PMNs out of blood vessels towards the irritant, attachment to microorganisms, ingestion and intracellular killing.

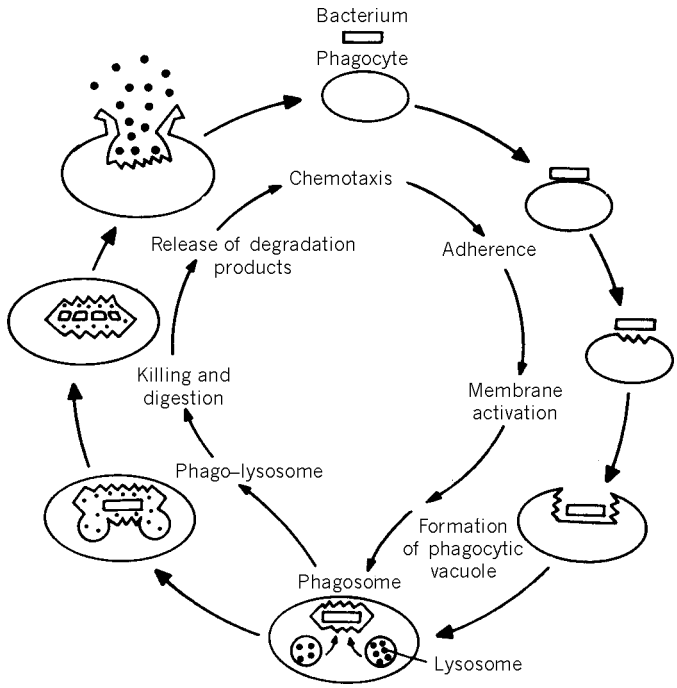


Figure 2.2. Phagocytosis: a multiphasic act.

Pattern Recognition and Ingestion

Phagocytic cells are among the most important cells endowed with a variety of receptors capable of recognizing molecular patterns expressed on the surface of pathogens or pathogen associated molecular patterns (PAMPS); these are shared by a large group of infectious agents and are clearly differentiated from 'self' patterns. Most body defence cells have **pattern-recognition receptors** (PRRs) for common pathogen-associated molecular patterns (Figure 2.3) and so there is an immediate response against the invading microorganism. Pathogen-associated molecular patterns can also be recognized by a series of soluble pattern-recognition receptors in the blood that function as **opsonins** and initiate the complement pathways. In all, the innate immune system is thought to recognize approximately 10^3 molecular patterns.

Most of these pattern recognition receptors (PRRs) are lectin-like and bind to externally displayed microbial sugars. They are glycoprotein in nature and are also known as **toll-like receptors** and are found on the surface of various body defence cells. They are so named because they recognize and bind to pathogen-associated molecular patterns - molecular components associated with microorganisms but not found as a part of eukaryotic cells. These include bacterial molecules such as peptidoglycan, teichoic acids, lipopolysaccharide, mannans, flagellin, pilin, and bacterial DNA. There are also pattern-recognition molecules for viral double-stranded RNA (dsRNA) and fungal cell wall components such as lipoteichoic acids, glycolipids, mannans, and zymosan. Binding of the microbial molecule to the toll-like receptor sends a signal through the cytoplasm to the nucleus of the cell where it activates genes coding for the synthesis and secretion of cytokines.

There is evidence that an adherent particle may initiate ingestion by activating an actin-myosin contractile system which extends pseudopods around the particle. The particle is eventually enclosed completely in a vacuole - the phagosome. Lysosomal granules come into contact and finally fuse with the phagosome forming a phagolysosome. Several hydrolytic enzymes are now released into the phagolysosome which act optimally at a low pH.

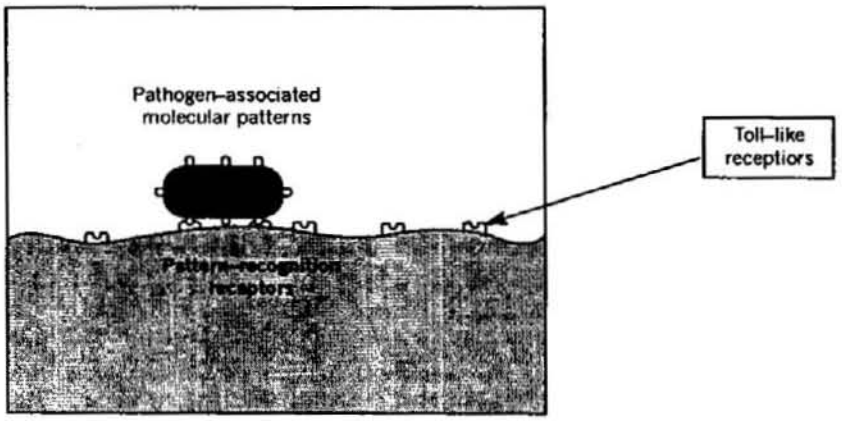


Figure 2.3. Pathogen-Associated Molecular Patterns Binding to Pattern-Recognition Receptors on Defence Cells.

Intracellular Killing

Intracellular killing utilizes two mechanisms:

(*i*) Oxygen dependent mechanisms and

(*ii*) Oxygen independent mechanisms (Table 2.1).

Table 2.1: Oxygen Dependent and Independent Mechanisms

Oxygen dependent mechanisms			
Glucose + $NADP^+$	hexose monophosphate shunt	pentose phosphate + NADPH	O2 burrst + generation of superoxide anion
NADPH + O_2	cytochrome b_{-245}	$NADP^+ + O_2$	
$2O_2 + 2H^+$	spontaneous dismutation	$H_2O_2 + {}^1O_2$	Spontaneous Formation of further microbicidal agents
$O_2 + H_2O_2$		$OH + OH^- + {}^1O_2$	
$H_2O_2 + Cl^-$	myeloperoxidase	$OCl^- + H_2O$	Myeloperoxidase generation of microbicidal molecules
$OCl^- + H_2O$		${}^1O_2 + Cl^- + H_2O$	
$2O_2 + 2H^+$	superoxide dismutase	$O_2 + H_2O_2$	Protective mechanisms used by host + many microbes
$2H_2O_2$	catalase	$2H_2O + O_2$	
Oxygen independent mechanisms			
Cationic proteins (incl. cathespin G)		Damage to microbial membranes	
Lysozyme		Splits mucopeptide in bacterial cell well	
Lactoferrin		Deprives proliferating bacteria of iron	
Proteolytic enzymes variety of other hydrolytic enzymes		Digestion of killed organisms	

Oxygen-dependent mechanisms

With the formation of the phagolysosome, there is a dramatic increase in activity of the hexose monophosphate shunt, increased glycolysis and increased oxygen consumption with an exaggerated formation of hydrogen peroxide, lactic acid and a subsequent fall in pH – a

prerequisite for optimal functioning of hydrolytic enzymes. Collectively the stimulation of all these pathways is called a "respiratory burst". The hexose monophosphate shunt generates NADPH, which is ultimately utilized to reduce molecular oxygen bound to cytochrome, causing a burst of oxygen consumption. As a result oxygen is converted to superoxide anion (O_2), hydrogen peroxide, singlet O2 ($^1O_2$) and hydroxyl radicals (OH) – all of which are powerful microbicidal agents. Furthermore the combination of peroxide, myeloperoxidase and halide (Cl^-) ions constitutes a potent halogenating system capable of killing both bacteria and viruses.

Killing by Nitric Oxide

Nitric oxide is known to be a physiologic mediator similar to factors that relax the endothelium. It is formed within most cells particularly neutrophils and macrophages and generates a powerful antimicrobial effect. It is thought to be particularly effective against *Salmonella* and *Leishmania spp.*, pathogens known to live comfortably within the cell and yet escape phagocytic killing.

Oxygen independent mechanisms

As a result of the oxygen dependent mechanisms the pH of the vacuole rises so as to allow several cationic proteins which are microbicidal to act optimally. These molecules are known as α-defensins and act selectively on microbial lipid components. Other substances such as the neutral proteinase (cathepsin G) are also powerful microbicidal agents. Lysozyme and lactoferrin also constitute bactericidal or bacteriostatic factors which are oxygen independent and can function under anaerobic conditions. Finally killed organisms are digested by hydrolytic enzymes and degraded products released to the exterior.

Extra-cellular Killing

Natural killer cells are large granular lympocytes. Their main role is to kill virus infected cells, this they do by secreting a cytolysin called perforin that attacks the membrane of the infected cell.

Large parasites such as helminths cannot be physically phagocytosed. Extra cellular killing by **eosinophils** (using the complement pathway) has evolved as a way of defence against these parasites. Remember eosinophilia can sometimes be an indirect clue that the patient has a parasitic infestation.

Soluble (Humoral) Bactericidal Factors

Of the soluble bactericidal substances elaborated by the body, perhaps the most abundant and widespread is the enzyme, **lysozyme** a muramidase which splits the peptidoglycan of the bacterial cell wall. **Human β-defensins** are proteins that play an important role in defending against microbial invaders along mucosal tracts.

There are also a number of plasma proteins collectively called the "**acute phase proteins**" which show a dramatic increase during infection. These include **C-reactive protein (CRP), serum amyloid A, α-1 antitrypsin, mannose-binding protein, fibrinogen** and **caeruloplasmin.**

During infection, microbial substances such as endotoxins stimulate the release of **interleukin**-1 (IL-1)-an endogenous pyrogen. IL-1 in turn induces the liver to release more CRP. The prime function of CRP is to bind to a number of micro organisms (in a calcium dependant fashion) which contain phosphorylcholine. This then enhances activation of

complement and thereby induces the acute inflammatory response. CRP therefore acts as an opsonin, coating organisms and triggering complement mediated lysis (see Chapter: 7). **Interferons** are anti-viral agents synthesized by cells that are infected by viruses. They are secreted into the extra-cellular fluid where they bind to receptors on uninfected cells. The bound interferon exerts an antiviral effect and prevents the uninfected cell from becoming infected.

However, many of these remarkable defence mechanisms are powerless in the face of overwhelming infection. Experience with chronically ill or debilitated patients indicates that many of these patients become "secondarily" infected — a reflection of the waning innate or natural defence mechanism in the host. Should innate immunity fail for some reason, all is not lost; other strategies of defence, far more powerful and exquisitely precise are brought into play, in the form of **adaptive or acquired specific immunity**.

CHAPTER – 3

IMMUNOBIOLOGY

The cellular organelles of defence in the human body are found in, what is collectively termed, the lympho-reticular system. This is broadly classified into

Internal	***External***
Blood	Respiratory tract
Tissues	Gastro-intestinal tract
Thymus	Genito-urinary tract
Lymph nodes	
Spleen	

The cellular constituents of the lympho-reticular system are:

• Phagocytic cells	Polymorphonuclear neutronphils
	Mononuclear phagocytes
	Eosinophils
• Lymphocytes	

The Polymorphonuclear Neutrophil (PMN)

This cell has a common haemopoietic stem cell precursor and is the dominant white cell in circulation. It is a non-dividing short lived cell with a multilobed nucleus and an array of granules. The neutrophil granules are of three types, the primary azurophilic granule contains myeloperoxidase, some lysozyme and a family of cationic proteins. The secondary granules hold lactoferrin, lysozyme and a B_{12} binding protein. The tertiary granules are the conventional lysozymes with acid hydrolases. Metabolism by glycolysis enables the cell to function under anaerobic conditions. The polymorphs provide a major defence against pyogenic bacteria- hence they are often loosely called "pus cells".

The Mononuclear Phagocyte

These cells are derived from bone marrow promonocytes. They enter the circulation as blood **monocytes** and finally settle in the tissues as mature **macrophages**. They constitute the **mononuclear phagocyte system (MPS)**. They are present throughout the connective tissue and around the basement membrane of small blood vessels. They are particularly abundant in the lung as **alveolar macrophages** and in the liver as **Kupffer cells**. They are strategically placed in the lining of spleen sinusoids and lymph node medullary sinuses to filter foreign

material. **Mesangial cells** in the renal glomerulus, **microglia** in the brain and **osteosclasts** in bone are also part of the MPS. Unlike polymorphs, they have a long life span. The macrophages feature predominantly in combating intracellular bacteria, viruses and protozoa. Mononuclear phagocytes express a myeloid receptor (CD14) which serves as a recognition molecule for a wide variety of bacterial envelope molecules, such as LPS from Gram negative organisms and components of Mycobacterial and Gram positive cell walls. Ligation of this receptor leads to macrophage activation.

Broadly, the macrophage serves two major functions : to ingest and destroy particulate matter– a function greatly enhanced when the foreign matter is coated by complement or antibody. The term opsonin is used to describe this coating with both antibody and complement to facilitate phagocytosis. The other function involves the initial recognition, processing and presentation of antigen to the T-cell to elicit the specific immune response.

Eosinophils

Large parasites such as helminths cannot physically be phagocytosed and extracellular killing by eosinophils is largely the mode of defence involved. These cells have distinctive granules which stain well with acid dyes. A **major basic protein** (MBP) is localized in the core of the granules, while an eosinophilic cationic protein together with a peroxidase have been identified in the granule matrix. Other enzymes found in the eosinophil include arylsulphatase B, phospholipase D, and histaminase. They have receptors for complement component C3b (see Chapter 7) and when activated produce a “respiratory burst” and concomitant generation of active oxygen metabolites. Eosinophils also produce a “perforin” like protein which can produce membrane damage via transmembrane plugs.

Most helminths activate the alternative complement pathway (see Chapter 7) and hence C3b is deposited all along the helminthic membrane. This allows for adherence of eosinophils through their C3b receptors. Upon activation, the eosinophil then launches its extra cellular attack which includes release of MBP and cationic proteins both of which damage parasite membranes.

Lymphoid Organs, Lymphocytes and Lymphocyte Traffic

Any discussion on lymphocytes takes its origins from a detailed study of the lymphoid organs. The immune system consists of a number of lymphoid organs, classified commonly as Primary or central and Secondary or peripheral organs. The primary organs are the thymus and the bone marrow; the secondary organs are the spleen, lymph nodes and the aggregates of lymphoid tissue in the respiratory, gastro-intestinal and genito-urinary tracts.

Lymphocytes derive from stem cells. Initially stem cells arise from the yolk sac and the foetal liver, but later in perinatal development, some originate in the bone marrow. Stem cells differentiate into lymphocytes in the primary lymphoid organs, namely the thymus and the bone marrow (Figure 3.1). Classification of lymphocytes on he basis of surface markers make use of two important classes of characteristics. One is known as cluster designation (CD) and the other the antigen recognition receptors. CD antigens represent families of surface antigens that can be recognized by specific antibodies produced against them. Thus mature T cells have CD3, CD4 or CD8 markers, and B cells have CDs 19-22. There are more than 200 distinct CDs.

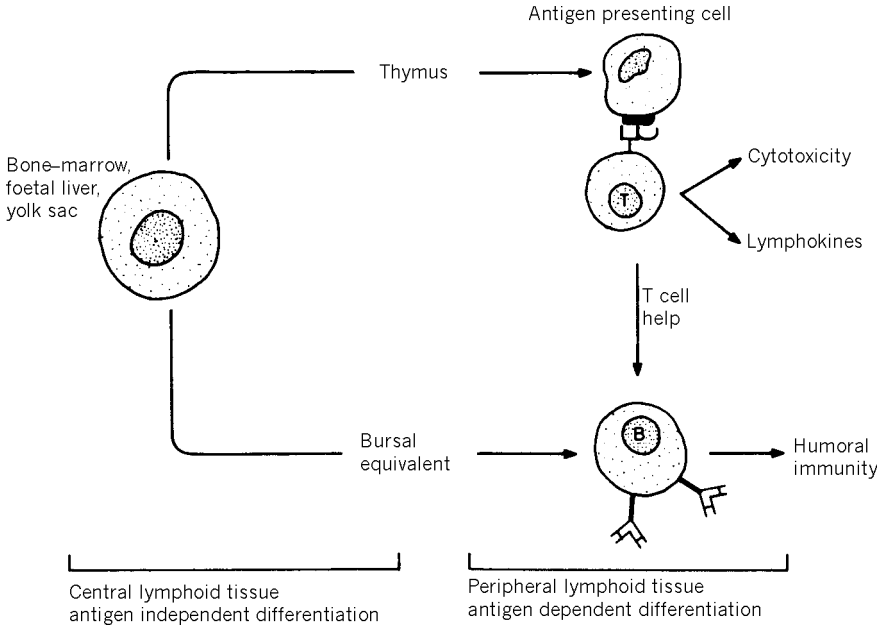


Figure 3.1. A simplistic overview of the immune system. * Bursal equivalent; since B cells were known to originate in the Bursa of Fabricius in birds.

The Primary Lymphoid Organs

The Thymus

The thymus is responsible for the development of T-dependent lymphocytes. It plays an important role in immunogenesis in the young and the T cells derived from it orchestrate the immune response throughout life. This central lymphoid organ differs from other lymphoid tissues. All other lymphoid tissues, are strategically placed to meet foreign particles, the thymus is protected from antigen contact. Furthermore, the rate of mitotic activity is greater than in any other lymphoid tissue and yet, the number of cells leaving it are comparatively less. The assumption is that a large number of cells made in the thymus die within its substance. This probably represents a system of homeostasis.

The thymus consists of two lobes surrounded by a thin capsule which extends into the substance of the gland to form septa–with the resultant formation of lobes and lobules. Peripheral portions of the lobule are heavily infiltrated with lymphocytes. Central portions have fewer lymphocytes and more epithelial cells.

The thymus is believed to perform two main functions, production of lymphocytes in the cortex and production of humoral substances in the medulla. These humoral substances (one of which is called thymosin) may induce differentiation of lymphocytes directly within the thymus or may control differentiation in the periphery. The thymus does not ontain any plasma cells. Unlike other lymphoid organs it contains two tissue types: lymphoid and epithelial. Some interdigitating cells, derived from precursors in the bone marrow, are also found in the thymus. These cells are rich in major histocompatibility antigens (MHC), which they display as surface markers. Thymic epithelial and interdigitating cells also influence T cell differentiation. Following infiltration of the thymus with pluripotential stem cells from yolk sac, foetal liver or

spleen, these cells acquire new surface antigens as they undergo differentiation. During intra thymic maturation thymocytes lose or retain certain surface markers.

Stage I thymocytes express a specific antigen termed CD2 on their surface. They also ontain an activated gene known as the T gene, which forms part of an antigen binding receptor on the T cell. As the thymocytes pass to **Stage II** they exhibit CD5 CD4 and CD8 surface markers, in addition, the gene that encodes for the β–chain of the T cell receptor becomes activated. Stage II cells then lose the CD5 marker and differentiate further into one of two types of a **Stage III cell**. Those that lose the CD8 surface marker become mature CD4+ T cells, whereas those that lose the CD4 marker become CD8 T cells (Figure 3.2).

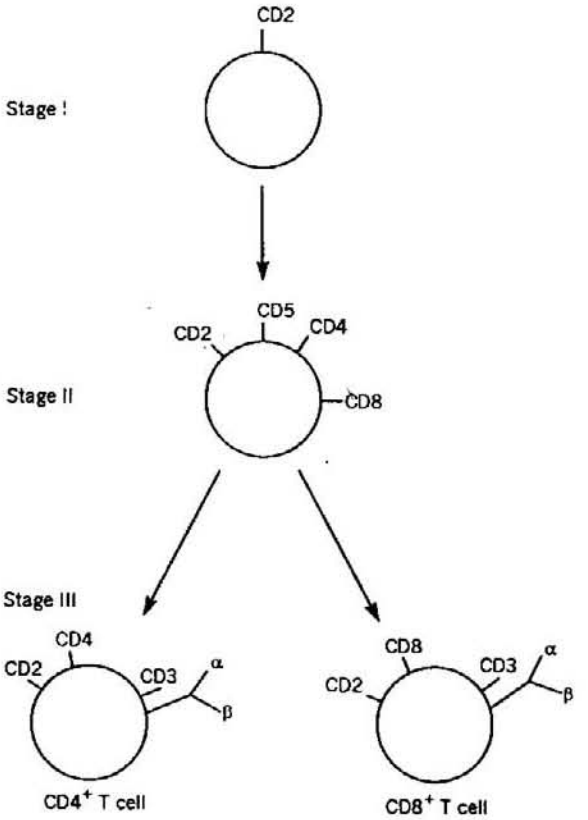


Figure 3.2. Stages of T cell differentiation.

T cell receptor genes become activated in both types of stage III cells, allowing for the expression of the complete T cell antigen binding receptor complex (CD3–α –β). During the process of maturation the T cells become immuno-competent : they learn to bind to specific antigens, when these antigens are presented in association with MHC molecules. Hence they are schooled to recognize MHC antigens during thymic maturation. In addition, T cells learn to differentiate between self and non self antigens. The T cells go through a selection process in the thymus based upon the **T cell receptor** that they posses. T cells that recognize self antigens are destined to die by **apoptosis** or programmed cell death. Finally, there are two separate populations of Stage III thymocytes released into the blood stream. These two populations are called : T-helper inducer and T-cytotoxic suppressor T cells. In the blood stream they are

carried to the lymphoid system's peripheral organs where they reside in thymus dependent regions of the peripheral lymphoid system.

The Bursal Equivalent and Bone Marrow

Birds have another primary lymphoid organ in addition to the thymus, situated near the cloaca, it is called the Bursa of Fabricius. The bursa is derived from gut epithelium in the embryo. Stem cells enter the Bursa of Fabricius where they differentiate into B cells capable of producing antibody. Bursectomized birds are completely B cell deficient though T cell functional activity is unaffected. There does not appear to be an organ in mammals that is equivalent to the Bursa of Fabricius. Stem cells differentiate into B cells in the bone-marrow and in the peripheral lymphoid organs themselves.

Secondary Lymphoid Organs

Lymph nodes

Cells and molecules are brought to the lymph nodes via afferent lymphatics (Figure 3.3). Lymphocytes move through the sinuses and leave the lymph nodes through efferent lymphatics. A great majority of lymphocytes that traverse through the lymph nodes come from the blood stream. A small percentage is generated in the lymph node from precursor cells. Most of the blood lymphocytes are T cells which migrate in and out of lymph nodes. A few B cells also come from the blood stream and many localize in the germinal centers of the cortex. The germinal centers are hence peripheral bursal equivalents. When lymph nodes have been stimulated by antigen, germinal centers increase in size and thereafter contain many lymphoblasts and is the site of memory B cell proliferation.

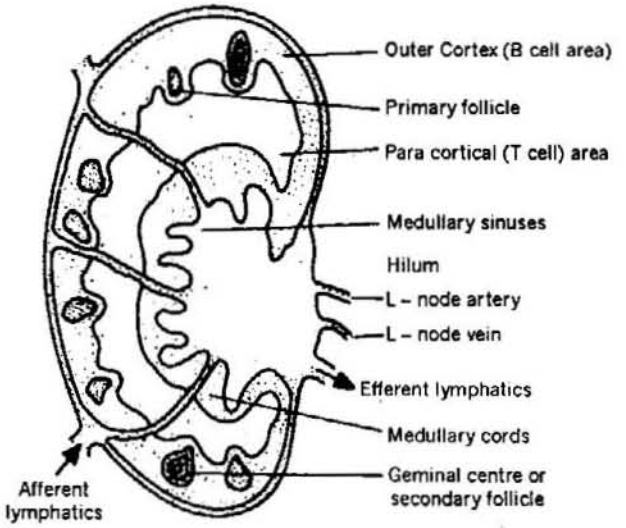


Figure 3.3. Cut section of lymph mode.

Cells of the dendritic cell lineage involved in T cell stimulation are bone marrow derived. In the skin they are known as **Langerhans cells** . These cells efficiently process antigen but cannot present it to T cells. Langerhans cells pick up antigen in skin and carry it

via **afferent lymphatic vessel**s to lymph nodes. Dendritic cells in lymph are known as "veiled" cells. In lymph nodes the cells, now known as **tissue dendritic cells** or **interdigitating cells**, may efficiently present antigen. The para cortical areas contain these interdigitating cells bearing MHC class II molecules complexed to foreign antigen. They present antigen to T cells which abound in the paracortical and sub cortical areas of the lymph mode. T cell function is closely associated and restricted by the presence of MHC antigens on antigen presenting cells.

Follicular **dendritic cells** are found in germinal centres. They are called dendritic because of their morphology rather than any lineage relationship with dendritic cells. In fact, there is considerable uncertainty about their developmental origin. They display C3 complement and IgG (Fc) receptors. Their function appears to entrap antigen as an antigen- antibody- C3 complex, and present it to B cells, as a stimulus to antibody production. They cannot present antigen to T cells but are important in developing responses by B cells.

The lymph nodes hence appear to be compartmentalized - germinal centers with immobile B cells, para cortical areas with migrant T cells. In addition, there are sinuses full of macrophages and a reticular network of dendritic cells that tends to hold antigen for a long time. All this appears to facilitate interactions between the different types of cells that are required for generating an immune response.

Spleen

In immunological terms the white pulp of the spleen is the bursal equivalent and is the store house for B cells. The red pulp which surrounds the white is a thymic dependant area housing T-cells (Figure 3.4).

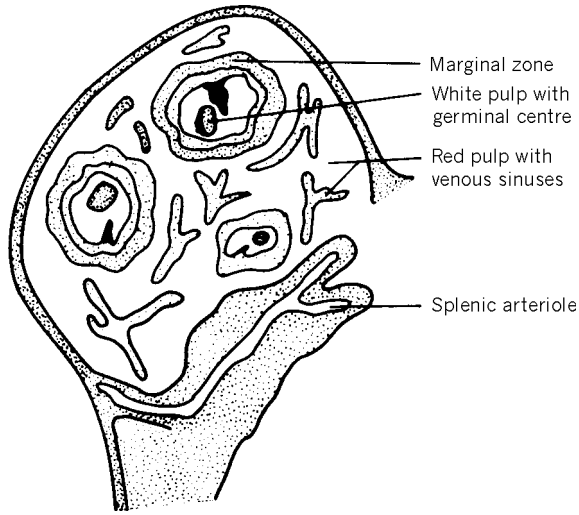


Figure 3.4. Cut section of spleen.

Other Lymphoid Tissue

The respiratory, alimentary and genito-urinary tracts are guarded immunologically by sub epithelial accumulations of lymphoid tissue which are not constrained by a connective

tissue capsule. These may occur as diffuse collections of lymphocytes, plasma cells and phagocytes in the lung and in the lamina propria of the intestinal wall, or as clearly organized lymphoid follicles. This includes the tonsils, the Payers patches and the appendix, lymphoid tissue found in the respiratory and urinary tracts: collectively they are termed, **mucosal associated lymphoid tissue (MALT)**. It is believed that MALT forms a separate interconnected secretory system within which cells committed to IgA or IgE synthesis may circulate.

Lymphocyte traffic

There are three major types of lymphocyte circulation :

(*a*) The seeding of stem cells from the foetal liver or bone marrow in the primary lymphoid organs and the subsequent differentiation and distribution of these cells to the peripheral lymphoid system,

(*b*) The recirculation of lymphocytes from blood to lymph to blood and

(*c*) The distribution of effector cells to particular parts of the body.

Immunocompetent cells involved in the immune response

The primary cells involved in the immune system are the lymphocytes. Functionally, lymphocytes are divided into

(*a*) T cells: These cell types regulate the immune response, are involved in cell mediated immune reactions and induce B cells to produce antibody.

(*b*) B cells: These cells differentiate into antibody producing **plasma cells**.

In addition there are a heterogenous group or groups of lymphocytes that are neither T or B cells. Morphologically these cell types cannot be differentiated. Binding of monoclonal antibodies is currently the most specific technique used to identify specific sub sets.

Sub sets of T cells

Depending on the presence of surface markers (CD4 and CD8) T cells are broadly and functionally divided into **helper T cells** bearing the marker CD4, **cytotoxic T** cells (bearing the CD8 marker).

Large granular lymphocytes

A portion of the lymphocytes circulating in blood lack the surface antigens of T or B cells. These cells are sometimes called **null cells**. A majority of null cells are **natural killer (NK) cells**. They are large granular lymphocytes that, in vitro, can kill a number of tumour cell lines in a non specific manner. For a comprehensive classification of lymphocytes, their subsets and functions see Table 3.1.

Phylogenetic evidence suggests that the development of immunologic competence coincides with the development of the lymphocyte. Using conventional stains the lymphocyte is a morphologically featureless cell. Functionally however, these cells play pivotal roles in the immunological response to foreign antigen.

Table 3.1: Classification of lymphocytes

Lymphocytes and their sub-sets	Main cell surface markers	Restrictions	Functions
T cells			
T helper cell	CD4+; CD3- T-cell receptor; CD2+	MHC- Class II	Stimulate B cell to produce antibody; Induce CD8+ T-cell cytokine secretion; macrophage activation
T cytotoxic cell	CD8+; CD3- T-cell receptor; CD2+	MHC- Class I	Lyse antigen bearing target cell
B cells			
Plasma cell and Memory B-cells	Ig+; CD19; CD20; CD21; CD23;	Antigen specific	Differentiate into antibody producing and memory cells
Large granular lymphocytes			
Null/ NK cells	CD 56+	Not MHC restricted	Kill tumour cells and show antimicrobial activity (mainly antiviral)

CD: Cluster differentiation designation

CHAPTER – 4

ANTIGENS AND IMMUNOGENICITY

The terms **antigen** and **immunogen** are often used synonymously. However, these terms antigen and immunogen, imply two closely related entities. One which describes a molecule that provokes an immune response is called an immunogen and the other describes a molecule which reacts with the antibody produced or with the activated cellular constituents of cell mediated immunity, is referred to as an antigen.

In contrast to this is the hapten. **Haptens** are small well defined chemical groupings such as dinitrophenyl (DNP) which are not immunogenic on their own but will react with preformed antibodies. To make a hapten immunogenic it must be linked to a carrier molecule which is itself immunogenic.

Antigens are recognised not only by antibodies but also by antigen specific T cell receptors. In contrast to immunoglobulins, which usually recognize intact antigen, T cell surface receptors recognize **processed antigen** on the surface of antigen presenting cells, together with the major histocompatibility complex (MHC) Class I or Class II surface proteins (refer chapter 10).

Antigenic Determinants and Epitopes

The part of the antibody molecule which contacts the antigen is termed the **paratope**. Consequently, that part of the antigen molecule that makes contact with the paratope is called the **epitope**. As most antigens are protein in nature they exist in a folded, three dimensional, tertiary structure. Hence there may be a cluster of amino acid sequences on the three dimensional structure constituting a series of epitopes. Each of these epitope clusters is what is meant by an **antigenic determinant**. An illustration of the antigenic determinants on bacterial cells is shown in Figure 4.1 a and b.

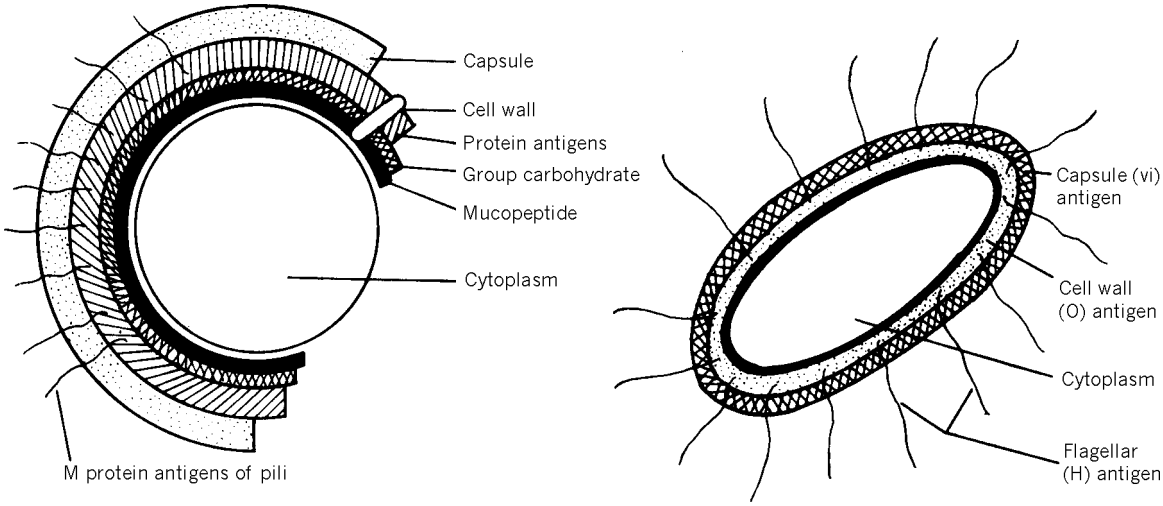


Figure 4.1. Antigenic determinants on a single bacterial cell.

Requirements for immunogenicity

The first and primary requirement for any molecule to qualify as an immunogen is that the substance be **genetically foreign** to the host. Sometimes body constituents are recognized as foreign leading to autoimmune disease. Normally the body discriminates self from non self. Certain particles do not elicit an immune response – they merely undergo phagocytosis, for example, carbon as coal dust.

Molecular size determines immunogenicity to some extent. The general rule is that particles with a molecular weight less than 10,000 are only weakly immunogenic or not immunogenic at all. The most potent immunogens are macromolecular proteins with molecular weights of 10,000 and more.

An immunogenic molecule needs to possess a certain degree of **chemical complexity**. It is difficult to establish a definite threshold, however, the general rule holds. Only pure lipids are non immunogenic. A solution of monomeric proteins may actually induce tolerance, but is highly immunogenic in the polymeric state. Several immunogens that do not induce an immune response in the pure form, do so when they are part of a larger particle. Hence, adjuvants are used to enhance immunogenicity.

Experiments have shown that **conformation** of a molecule could be important to its antigenicity. For example, the lysozyme molecule is a good antigen in its native form which consists of several amino acids folded into a loop with the aid of a disulphide bond. If the disulphide bond was disturbed, so that the loop conformation was no longer present, the antigenicity of the molecule would be dramatically reduced.

Studies have also shown that the **amino acid sequence** is important to antigenicity. Certain molecules that have no apparent complexity in structure, can serve as antigens provided certain short, but crucial stretches of amino acids are present in an undisturbed fashion.

Other workers have shown that the **mobility of a segment** of the antigen molecule influences its antigenicity. Using the tobacco mosaic virus, researchers have found that certain segments with high mobility (movement of 1 angstrom from the protein back bone position) were more antigenic than non mobile segments.

Parts of the peptide chains which **protrude significantly** from the globular surface tend to be sites of high epitope density. Hence **accessibility** of these high epitope density areas to the recognition system determines the outcome of the immune response (Figure 4.2).

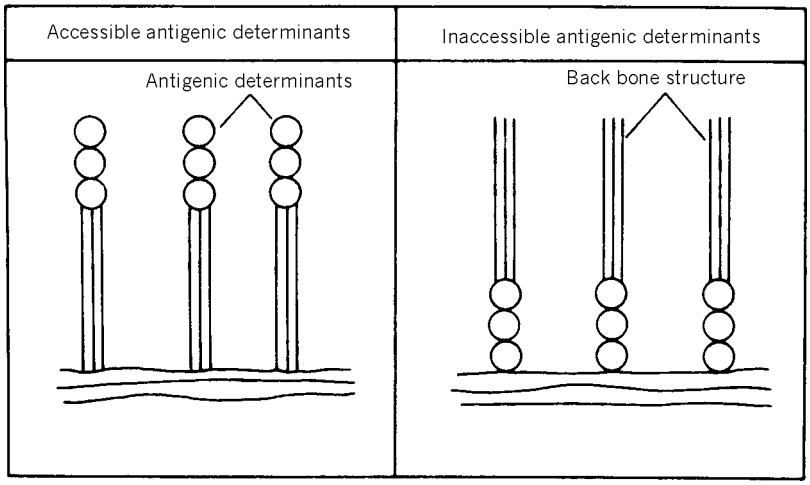


Figure 4.2. Accessibility of antigenic determinants.

Antigens and antibodies interact by **spatial complementarity** and not by co-valent bonding, and like enzyme – substrate interactions, can be readily reversed. The idea that antibody recognizes antigen through complementary shapes on paratope and epitope is simplistically illustrated using the "lock and key" analogy (Figure 4.3).

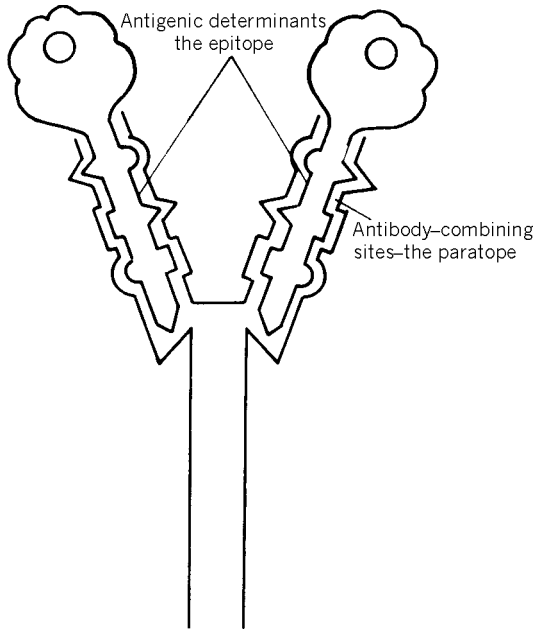


Figure 4.3. Antigen and antibody interact by spatial complementarity; the lock and key analogy.

The Forces that Bind Antigen to Antibody

The forces that bind antigen to antibody become larger as intercellular distances decrease. These forces are, in essence, no different from the 'non-specific' interactions which occur between any two unrelated proteins. These intermolecular forces are:

- electrostatic (attraction between oppositely charged ionic groups)
- hydrogen bonding (reversible hydrogen bridges between hydrophilic groups)
- hydrophobic bonding (similar to the manner in which oil droplets in water merge to form a single large drop, side chains of valine, leucine and phenylalanine tend to associate in an aqueous environment. This may account for over 50% of the strength in an antigen antibody bond).
- Van der Waals forces : interaction between the electrons in the external orbits of the two different macro molecules.

At this point of the discussion, there are two commonly used terms that need definition:

(*i*) **affinity** or strength of binding of antigen to antibody. The term affinity alludes to the strength of the bond between antigenic determinant and monovalent antibody. Hence those antibodies that fit closely to an antigenic determinant and are not easily dissociable are known as high affinity antibodies.

(*ii*) **avidity** of antiserum for antigen refers to the overall strength of interaction between a large antigen with multiple epitopes and a polyvalent antibody molecule such as the IgM. Each one of the many interactions is by itself a low affinity one. This is a function of multivalency i.e. an antigen with several determinants will elicit a variety of antibodies as illustrated in Figure 4.4. Antiserum in Figure 4.4b is multivalent, binding is greater, dissociation is not easy and it is of higher avidity than antiserum 4.4a.

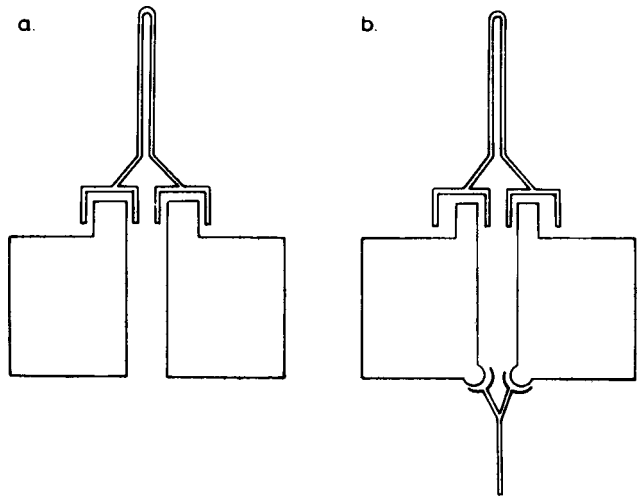


Figure 4.4. Avidity of antiserum for antigen a. monovalent and easily dissociated antiserum b. multivalent antiserum has a higher avidity.

Antigen-antibody Specificity is not Absolute

It is widely known that antigen-antibody reactions are exquisitely specific. An antibody raised to one determinant will not react with another determinant. However, in practice, the term **cross reactivity** is widely used. An antiserum raised against a given antigen can cross react with a partially related antigen which bears an identical or similar determinant (Figure 4.5).

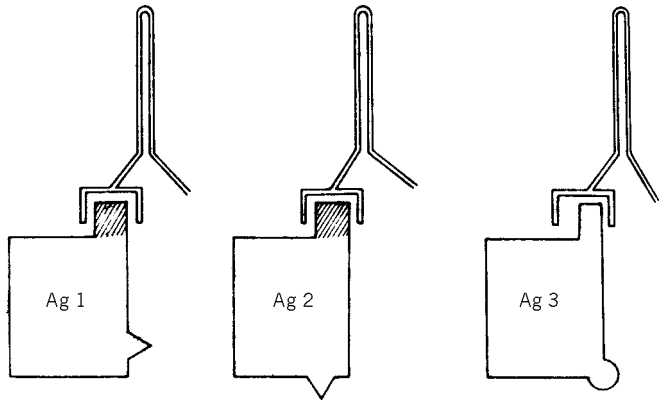


Figure 4.5. Cross reactivity of antibodies.

Antiserum raised to Ag1, will react with Ag2 due to the identical determinant (hatched) shared by the two antigens. Antiserum to Ag1 will also react weakly to Ag3 because, the determinants though not identical, are similar in shape.

Thus it is possible that each antibody will react not only with the antigen which stimulated its production, but also with some quite unrelated molecule bearing similar looking determinants.

The Heterophile Antibody Response

A number of similar antigen molecules are found in phylogenetically unrelated species. The cross reacting antigens of human heart tissue and the group A β-haemolytic *Streptococcus* is an example.

The **Forssman antigen** is another example which is found in the tissues of many species. This antigen is itself not found in man, however a great variety of other cells and tissues could sensitize man to this antigen. Following an attack of infectious mononucleosis, an infection by the Epstein-Barr virus, antibodies are produced which react not only to the virus but also to a completely unrelated antigen-the sheep red cell. This is known as the heterophile antibody response and forms the basis of the Paul-Bunnel test for infectious mononucleosis.

CHAPTER – 5

IMMUNOGLOBULINS I: STRUCTURE AND FUNCTION

The first real chemical information regarding the structure of antibodies was provided by **Tiselius and Kabat** in the early 1940s. These workers demonstrated that the fraction of serum proteins that migrated most slowly in electrophoresis contained most of the serum antibodies. In the 1950s **Porter** used proteolytic enzymes such as papain to cleave the antibody molecule in an attempt at defining its structure and function. Amino acid sequence of the IgG molecule was determined by **Edelman *et al*** in 1969.

In all these studies Bence Jones proteins which are monoclonal immunoglobulin light chains shed in plasma and urine from patients with plasmacytomas or multiple myelomas have been an invaluable source of large amounts of immunoglobulin molecules.

Basic Structure of the Immunoglobulin Molecule

Immunoglobulins are glycoproteins (Figure 5.1). The basic unit of the molecule is a four chain monomer. These four polypeptide chains consist of two identical heavy chains and two identical light chains, designated **H and L chains** respectively. Each polypeptide chain contains an amino terminal and a carboxy terminal. The amino terminal portion of the molecule is part of the **variable or 'V' regions** and the carboxy terminal portion the **constant or 'C' regions**. Hence the light chains have a variable (V_L) and a constant (C_L) region and so do the heavy chains have a variable region (V_H) and a constant region (C_H). The C_H region is further divided into three areas and these are designated CH_1, CH_2 and CH_3 in order from the amino end to the carboxy end of the heavy chain.

The part of the antibody molecule that binds antigen is formed only by small numbers of amino acids in the V regions of the H and L chains. The **hinge region** is formed in the two heavy chains between the first and second C regions. It is flexible and more exposed to enzymes and chemicals due to its high proline content, hence papain acts at the hinge region to fragment the immunoglobulin molecule. Digestion of the molecule by papain produces two Fab (antigen binding) fragments and one Fc (crystallizable) fragment, which lacks the ability to bind antigen. The enzyme pepsin strikes at a different point. It cleaves the Fc fragment from the remainder of the molecule, leaving the hinge region intact. It leaves behind a large fragment designated $F(ab)_2$ since it is still divalent with respect to antigen binding just like the parent antibody. The immunoglobulin molecule is linked together by several **disulphide bonds** from H to H chains, H to L chains and L to L chains.

Intrachain links also exist. However, the disulphide bonds are not really responsible for holding the chains of an immunoglobulin molecule together because removal of these bonds retains the intactness of the molecule. Besides, a few subtypes of immunoglobulin molecules do not have disulphide links between chains, yet retain the basic four chain structure. It has been shown that noncovalent forces (electrostatic, hydrogen bonding and Van der Waal's forces)

are far more important in maintaining the integrity of the molecule than the co-valent disulphide bonds. All immunoglobulins are measured using a **sedimentation coefficient (measured by Svedberg)** or **S value**. Higher the molecular weight of immunoglobulin, larger is the S value.

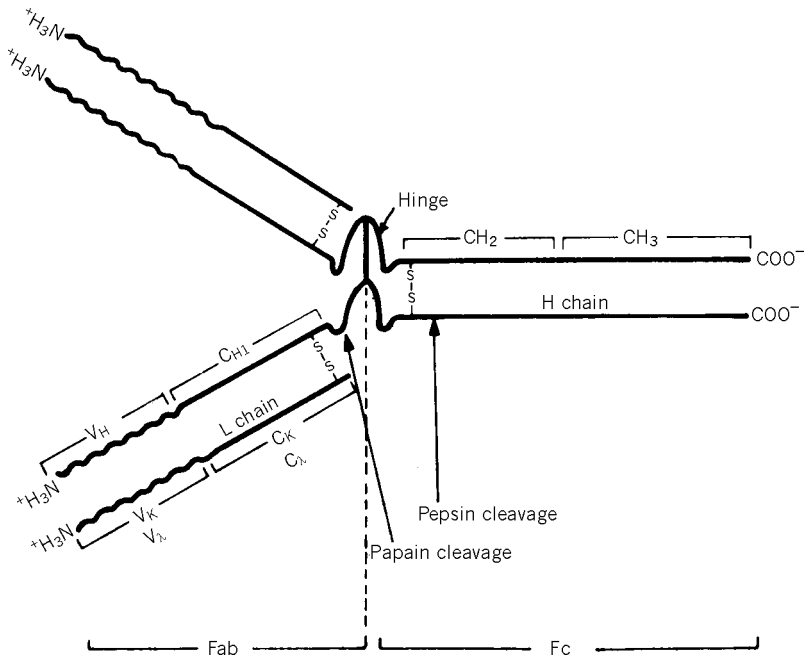


Figure 5.1. Structure of an immunoglobulin molecule.

Isotypes

Based upon the structure of their heavy chain constant regions, immunoglobulins are classed into major groups termed classes. These classes are designated IgG, IgM, IgA, IgE, and IgD. Since these classes are all variants of the immunoglobulin molecule they are termed isotypic variants or **isotypes**. The 5 different isotypes are defined by differences in the amino acid sequences of their constant regions and therefore by antigenic differences in the C_H regions of the molecule. The C_H region of the respective immunoglobulins are designated by greek alphabets. Hence IgG has γC_H region; IgM a μ; IgA an α; IgE an ε and IgD a δ type heavy chain constant region. IgG, IgA and IgM have been further divided into subclasses by relatively minor differences in their C_H regions. Therefore, an IgG1, IgG2, IgG3 and IgG4 have been described and similarly an IgA1 and IgA2 as well.

Since the immunoglobulin molecule is a protein structure with antigenic differences in the various isotypes, it is possible to raise an antiserum to any one isotype (say IgG) which can be absorbed to remove any cross reacting antibodies. This antiserum will then be capable of reacting with IgG but not with IgM, IgA, IgE or IgD.

Likewise the light chain constant regions also exist in isotypic forms known as κ and λ. Immunoglobulins possess either κ or λ light chains, never mixed, on a single molecule. Thus IgG exists as IgG κ or IgG λ , IgM as IgM κ or IgM λ and so on.

The Variable Regions and Heterogeneity of Immunoglobulins

The antibody population in any individual is incredibly heterogenous. In effect, this means that the normal immune system can generate an immunoglobulin for every possible immunogen that the system may encounter during its life time. It does this by constantly changing the amino acid sequences in the variable regions of the immunoglobulin molecule. Thus instead of carrying the enormous load of having to produce millions of billions of immunoglobulins to encounter every foreign agent, the immune system is equipped with the provision of rapidly changing and modifying the variable regions of immunoglobulins to suit a particular antigen. The terminal 110 amino acid sequences of the L and H chains constitute the variable or heterogenous regions of the immunoglobulin molecule. Certain sequences in the variable region show quite remarkable diversity and are termed **hypervariable regions**, hot spots or **complementarity determining regions (CDR)**. These hyper variable sequences have been localized to three segments each on the H and L chains. Not surprisingly, these areas of hypervariability are intimately involved in the formation of the antigen binding site.

Idiotypes

Just as the C regions of the various isotypes have antigenic determinants different from each other, idiotypes are formed based on antigenic determinants in the variable region that distinguish one V domain from the other. And just as an antisera can be raised to the different isotypes an anti idiotypic serum can be raised to the various idiotypes of immunoglobulin molecules. Such anti idiotypic sera are useful in detection and typing of the various idiotypes produced by the system. A startling realization resulting from research in this area was that an individual could make antibodies to his own idiotypes (auto anti idiotypic sera). The consequences of this will be discussed later in the Jerne net work theory. (Chapter 16)

Immunoglobulins in their natural state

In their natural state immunoglobulins are folded into a three dimensional structure. Significantly the hyper variable, antigen binding sequences all appear as loops at one end of the variable domain, clustered close to each other (Figure: 5.2). They are therefore in an ideal

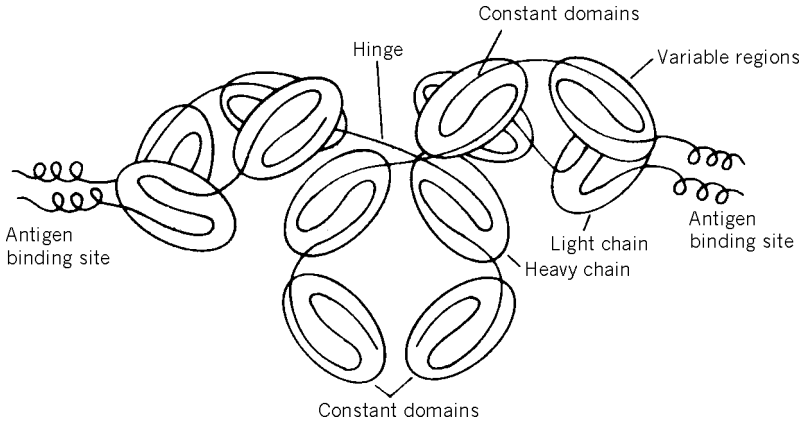


Figure 5.2. Characteristic folding pattern of the immunoglobulin molecule. An antibody molecule is a Y-shaped protein made of four polypeptide chains. Two heavy chains extend from the stem (blue), two light chains are confined to the arms (grey).

position to serve the function of antigen recognition and this has been confirmed by X-ray crystallographic analysis. Besides sequence heterogeneity, the hypervariable loops ensure tremendous diversity in combining specificity for antigen through variation in the shape and nature of the surface they create. (Figure 5.3)

Biological Role of the Constant Regions

If the variable regions function as antigen binding sites, what biological functions do the constant regions serve? The classes of antibody differ from each other in their ability to carry out certain secondary biological functions. For example, the CH_2 regions of IgG and IgM fix complement most efficiently; the CH_3 domain of IgG binds to Fc receptors on phagocytic cells, NK cells (natural killer) and placental syncytiotrophoblasts; also to Staphylococcal protein A, and the CH_3 region of IgE binds to Fc receptors on homologous mast cells and basophils.

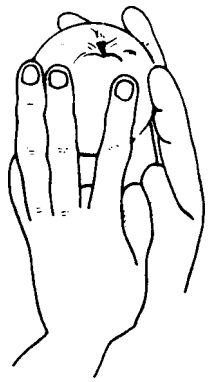


Figure 5.3. An analogy for the antigen binding site. The three fingers of each hand constitute the six hypervarible regions which form the antigen binding site. The apple is the antigen!

Immunoglobulin Classes and Subclasses

The following section describes the physical and biological characteristics of the 5 major immunoglobulin classes: IgG, IgD and IgE exist in the basic four chain structural form, IgM and IgA occur as complexes of this basic four chain unit.

Immunoglobulin G

IgG constitutes 75% of the total serum immunoglobulins in humans. During the secondary immune response it is the major immunoglobulin to be synthesized. Hence it plays a vital role in the defence against infection. Being the immunoglobulin that can cross the placenta in humans it is responsible for the protection of the neonate in the first months of life. This is reinforced by the presence of colostral IgG in breast fed infants. IgG diffuses readily into extravascular spaces and hence provides a major defence against bacterial toxins and other blood borne infectious agents. Organisms coated with immunoglobulin G attract macrophages via their Fc receptors: thus enhancing phagocytosis. Further, complexes of antibody and bacteria also activate complement thereby chemotactically attracting polymorphonuclear phagocytic cells and promoting phagocytosis, since “polymorphs” also possess receptors for the Fc portion of immunoglobulin and for complement components. The complement binding site on the IgG molecule appears to be in the CH_2 domain.

In a similar way the extra cellular killing of target cells coated with IgG is mediated largely through recognition of the Fc portion of the IgG by surface receptors on NK cells. Such Fc receptors are also present on platelets and when complexed may lead to release of vasoactive amines. IgG is unable to bind firmly onto most cells, but has the ability to bind to guinea pig skin - the significance of which remains unclear. As will be discussed later the property of the Fc portion of IgG to bind to protein A on the surface of *Staphylococcus aureus* has been greatly exploited for use in diagnosis and research.

Immunoglobulin M

IgM is the largest immunoglobulin in size (Figure 5.4). It exists as a pentamer of the basic four chain subunits held together by disulphide bonds. A relatively small molecule; the J chain participates in the polymerization of IgM via a sulphydryl residue near the carboxy terminal. The heavy chains of IgM are designated 'μ' chains.

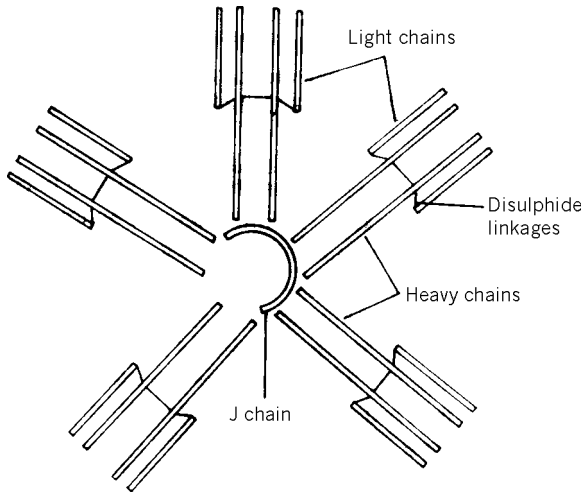


Figure 5.4. Structure of the *IgM* pentamer.

Electron microscopy studies reveal that it is shaped like a star, but when it is attached to a bacterium, its antigen binding sites are bound to the bacterial surface. This changes the appearance of IgM to a crab like form and causes cross-linking of the different antigenic determinants or epitopes on the bacterial cell surface by the polyvalent IgM molecule (Figure 5.5).

The μ chains bear an extra CH region; the IgM heavy chain therefore consists of regions CH_1 to CH_4. IgM antibodies tend to be of relatively low affinity but because of their valency they bind with high avidity to antigens with multiple epitopes.

IgM antibodies appear early in the response to infection and because of their size, are largely confined to the blood stream. They are an important defence mechanism against bacteria. The size and valency of IgM makes it a very effective agglutinating and cytolytic agent. This is so because it is the most efficient complement fixing immunoglobulin. IgM predominates in certain antibody responses such as the "natural" isohaemagglutinins (anti-A, anti-B) and to the typhoid 'O' antigen. Since IgM does not cross the placenta its presence in cord blood indicates active foetal infection, furthermore, since the IgM response is short lived its presence may be

helpful in establishing an acute infection. Monomeric IgM has a hydrophobic sequence stitched into the 'C' terminal of the heavy chain to anchor the molecule into the cell membrane of the B-lymphocyte. The anchored IgM is the major antibody receptor used by B-lymphocytes to recognize antigen.

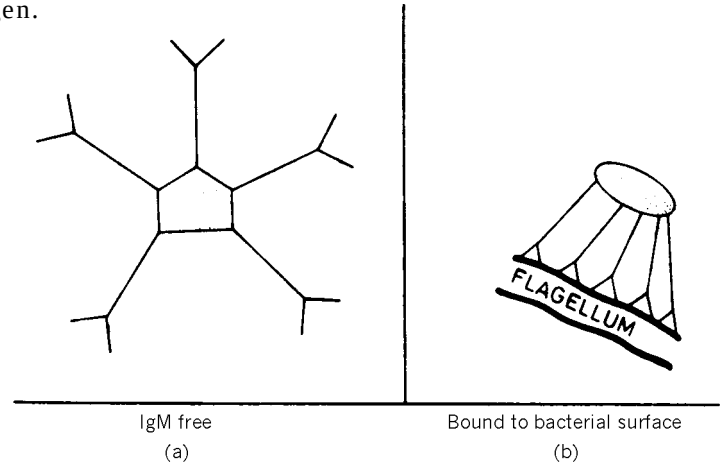


Figure 5.5. (a) IgM free and (b) bound to the bacterial surface.

Immunoglobulin A

IgA is actively secreted by, what is now known as, mucosal associated lymphoid tissue **(MALT)**. IgA appears selectively in sero-mucus secretions such as saliva, tears, nasal fluids, colostrum and in secretions of the lung, genito-urinary and gastro-intestinal tracts. It is present in these fluids as a dimer, stabilized against proteolysis by combination with another protein, the secretory component, which is synthesized by local epithelial cells and has a single peptide chain of molecular weight 60,000 (Figure 5.6). The IgA is synthesized locally by plasma cells and dimerized intra cellularly before secretion, with the help of a cysteine rich polypeptide called the J chain which has a molecular weight of 15,000. The dimeric IgA is released from the plasma cell and binds strongly to a secretory component precursor present on the surface of the glandular epithelial cell. It is actively endocytosed and transported within the endocytic vacuole to the mucosal surface. Cleavage of the receptor releases the IgA, still attached to part of the receptor: termed the secretory piece, into sero-mucus secretions (Figure 5.7).

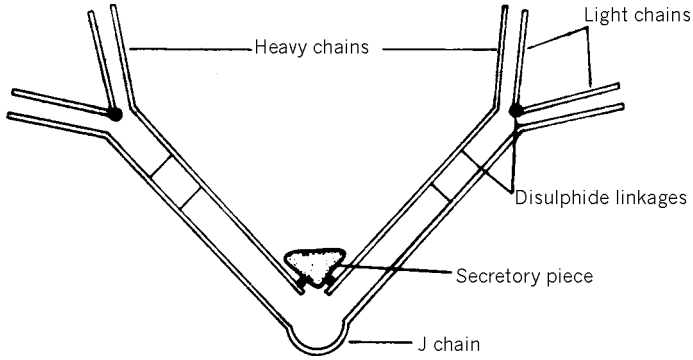


Figure 5.6. Structure of the IgA dimer.

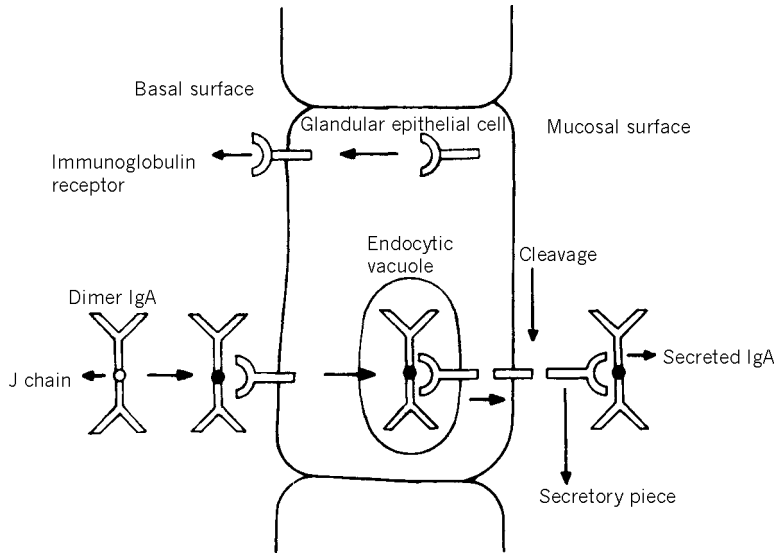


Figure 5.7. Formation of secretory *IgA*. The glandular cell on the mucosal surface synthesizes an immunoglobulin receptor inserted into the basal membrane. Dimeric *IgA* binds to this receptor, is endocytosed in a vacuole and transported to the mucosal surface. Cleavage of the receptor yields secretory *IgA* into the lumen with part of the receptor (as the secretory piece) still in place.

Since IgA is most abundant in body secretions it performs the role of defending the exposed external surfaces of the body against attack by micro organisms. IgA functions by inhibiting the adherence of coated micro organisms to the surface of mucosal cells thereby preventing entry into the body tissues. IgA activates complement via the alternative pathway but is unable to do so via the classical pathway. IgA does not cross the placenta, but contributes to the protection of the newborn by being in abundance in colostrum. IgA is the prime functional unit of the mucosal associated lymphoid tissue (MALT).

Immunoglobulin E

IgE is present in very low concentrations in serum. IgE antibodies have a high affinity for mast cells and binding occurs via the Fc portion of the immunoglobulin molecule. On contact with specific antigens called allergens, the mast cells undergo degranulation with release of vasoactive amines. This process is responsible for the wheal and flare skin reaction in allergy, for the symptoms of hay fever and extrinsic asthma. IgE also has the ability to attach to human skin where they are probably bound to mast cells. IgE is found mainly in the linings of the respiratory and gastro intestinal tracts, where they form constituents of MALT.

The main physiological role of IgE would appear to be protection of external mucosal surfaces where release of vasoactive amines could perpetuate the acute inflammatory response. Infectious agents penetrating the IgA defences would combine with specific IgE on the mast cell surface to trigger the release of vasoactive agents and other factors chemotactic for granulocytes. This would lead to an influx of plasma IgG, complement, polymorphs and eosinophils causing an effective inflammatory response. It is possible that IgE acts in this way as a defence against helminthic infections; which are characterized by an exaggerated IgE response.

Immunoglobulin D

This immunoglobulin has the basic four peptide structure. It is present in serum in trace amounts. Because of an extended hinge region it is relatively liable to degradation by proteolytic enzymes. The main function of IgD has not yet been determined; with IgM it is found abundantly on the surface of B-lymphocytes. It has been suggested that they may operate as antigen receptors and in the control of lymphocytic activation and suppression.

Immunoglobulin Sub-classes

IgG has four isotypic sub classes termed IgG1, IgG2, IgG3 and IgG4. The differences lie in the chains of the molecule. Certain functional and biological differences exist between the classes for eg: IgG3 does not combine with staphylococcal protein A, IgG2 does not cross the placenta readily, IgG4 does not bind to complement in the classical pathway nor does it bind to monocytes.

IgA exists as IgA1 (constitutes 80-90% of total IgA), IgA2 is unusual because it lacks H to L disulphide bonds.

Major characteristics of the immunoglobulin classes have been summarised in Table 5.1.

Table 5.1: Characteristics of the immunoglobulin classes

Characteristics	IgG	IgM	IgA	IgE	IgD
Sedimentation coefficient	7S	19S	11S	8S	7S
Molecular weight	150,000	900,000	160,000	200,000	185,000
Functions	Most abundant in extra-vascular spaces; combats micro-organisms and their toxins	Early in immune response. Effective in agglutination; first line defence in bacteraemia	Major Ig in sero-mucus secretions, defends external body surfaces	Protects external body surface, responsible for symptoms of allergy. Raised in parasitic infections.	Mostly present on surface of B lymphocytes
Complement fixation classical	++	+++	–	–	–
alternative	–	–	+	–	–
Cross placenta	+	–	–	–	–
Fix to homologous mast cells / basophils	–	–	–	+	–
Bind to macrophages and polymorphs	+	–	+	–	–

❖❖❖

CHAPTER – 6

IMMUNOGLOBULINS II: THE GENETICS OF ANTIBODY DIVERSITY

The immune system is clearly essential for survival; without it death from infection would be inevitable. The molecules of the immune system maintain constant surveillance against invading micro organisms. They recognize an almost limitless variety of foreign substances, they can distinguish and bind to every possible antigen that the host is likely to meet during its lifetime and some that it is not. Furthermore, they remember each infection so that a second exposure to the same organism is dealt with more efficiently. This implies that millions of species of antibody molecules need to be synthesized. Since an antibody is an assembly of protein chains, its structure is specified by a gene. It would appear that the genome must contain millions of antibody genes, since each antibody is different depending on the antigen that provokes its formation. Yet the total genetic complement of a mammal amounts to perhaps a million genes. How then does the immune system function on such a small defence budget demanding that only a small share of the genome and the body's resources be ear marked for the production of antibodies?

The paradox of a limited number of genes and an apparently limitless capacity to generate different antibodies has been a major puzzle for immunologists,and essentially two competing theories were put forward in explanation - the germ line and somatic recombination theories.

The Dreyer-Bennett Hypothesis

William Dreyer and Claude Bennett in 1965 working at the Caltech laboratories made a radical proposal. Instead of assuming that the genetic information for an antibody light chain is specified by a continuous array of codons (a codon is a triplet of nucleotides which codes for an amino acid), they proposed that there were several hundred stretches of DNA that coded separately for different kinds of variable regions and one gene coded for the constant region in the germ line DNA (DNA in the sperm cell and ovum). They also implied that these separate lists of information must somehow come together to form a contiguous, coherent message. This proposal initially attracted considerable criticism since it opposed the central dogma of protein biosynthesis; the one gene, one polypeptide theory. Dreyer and Bennett claimed that two or more genes could control the production of one polypeptide chain, at a time when no means of rearrangement of genes in germ line cells was known of. Yet their idea proved to be essentially correct.

The Generation of Antibody Diversity by Somatic Recombination

The DNA in the germ cells (in the male sperm and the female egg) or in the early embryo contains several bits and pieces of the gene that dictates antibody formation. These bits and pieces are shuffled in the DNA of the B-lymphocyte to yield the required antibody. The mechanisms responsible for shuffling of immunoglobulin DNA became clear when it was possible

to sequence fragments of DNA after they were cloned in bacteria. These experiments by **Tonegawa, Hozumi** and others showed that i) V and C genes were farther apart in embryonic cells than in antibody producing cells and ii) a particular light chain had a small segment of DNA coding for a part of the V region: this fragment was called the V segment. The rest of the V region was coded for by a stretch of DNA located thousands of base - pairs away. This interposed segment was called J for "joining". The C segment was also located about 1300 base pairs "upstream" and was found to code for the C region. Each light chain was assembled by combining the scattered V,J and C segments.

Thereafter it was discovered that there were a few hundred V segments in germ line DNA, each differing slightly in amino acid sequence, and four distinct J segments.

The potential diversity of the heavy chains is even greater. In addition to V and J segments the genes for the heavy chain variable segment include a third fragment designated D (for diversity). Mouse germ line cells have about 20 D segments. In principle, these segments can be brought together in over 10,000 combinations. Combining a light with a heavy chain can yield over 10 million distinct antigen binding sites.

The Assembly of a Functioning Immunoglobulin Gene

The assembling of a functional immunoglobulin gene takes place in two stages of a process called **Somatic Recombination**. As shown in Figures 6.1 and 6.2 the germ line DNA contains

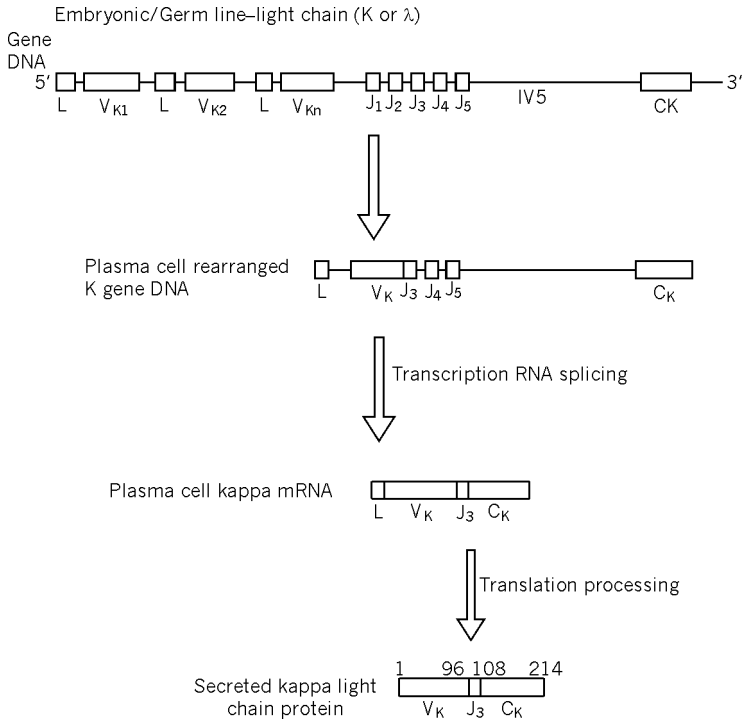


Figure 6.1. Organization of the human light chain gene; illustrates the process of kappa light chain secretion. The same principle holds for the lambda light chain. Multiple variable (Vk) region bits exist in the germ line DNA, each accompanied by a leader (L) sequence. There are 5 joining (J) segments coding for amino acids 96-108. There is only one constant (C) gene. DNA rearrangement joins 1 V region to 1 J region to the C region. The intervening sequences (IVS) are removed by RNA splicing. This yields one kind of high chain. Many other permutations and combinations are possible.

an array of segments from which permutations and combinations occur. Each B- cell DNA contains randomly selected V and J segments for a light chain or V,D and J segments for a heavy chain. These segments are fused by enzymes that delete all the intervening DNA, sometimes termed **intervening sequences (IVS)** or **introns**. Each of the V segments is preceded by a short coding sequence known as the "leader" sequence and this is thought to play a part in the transport of the antibody molecule through the cell membrane, it is then cleaved away as the antibody molecule passes across the membrane.

The second stage in this process relies on mechanisms of **RNA splicing**. An RNA transcript is obtained as shown in Figure 6.1, with the selection of a V, coding for amino acids(1-95), a J coding for amino acids 96-108 and the C gene separated from the J gene by an intron of 1250 nucleotides. After RNA splicing the mRNA contains a single V gene in combination with a single J which in turn tags on to a single C gene. All introns have been excised. This is then translated to form the protein molecule which is the antibody. In case of the heavy chain gene order the fusion order yields a V,D, J and C segments (See figure 6.2).

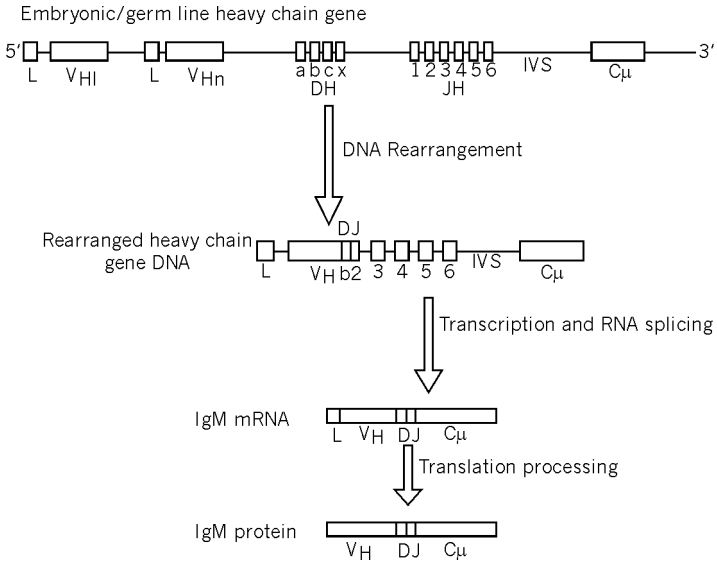


Figure 6.2. Organization of the human heavy chain gene. Similar to the k light chain V fragments, there are multiple variable (V_H)regions with preceding leader (L) sequences. There are the 6 functional joining (J_H) segments and in addition, a family of diversity (D_H) segments increase the variety of possible combinations between segments. Single VH, DH and JH regions recombine with each other at the DNA level. RNA splicing removes the intervening sequences (IVS) to join the constant (C_μ) to the rest yielding a secreted IgM protein - among the first immunoglobulins to be secreted by a stimulated plasma cell.

Fusion of gene segments in B- cell DNA is unusual and may even be unique to the immune system. It employs a set of enzymes that can bring together distant V,D and J segments deleting all the DNA that separates them. Certain signal sequences that guide the site of action of these splicing enzymes have been discovered. As shown in Figure 6.3, just down stream from the V gene of a kappa chain there is a distinctive pattern composed of a heptamer or seven nucleotide unit, followed by a spacer and a nonamer or nine nucleotide unit. Just upstream of the J segment there is a complementary nonamer-spacer-heptamer pattern. These units could provide a template for the enzymes to cut and rejoin the double helix. Similar signal sequences are found in the heavy chain genes.

Humans utilize lambda light chains in one third of their immunoglobulins.In contrast to kappa light chains, lambda light chains have multiple constant region genes arranged in tandem.

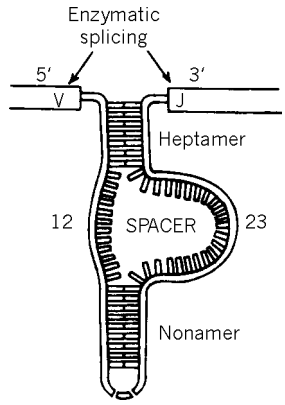


Figure 6.3. Sequences that facilitate V-J, V-D and D-J joining. The characteristic sequences form a base paired heptamer, a spacer of 12 bases (at the V end) and 23 bases (at the J end) and then a base paired nonamer. These characteristic sequences facilitate splicing by DNA repair enzymes bringing distant V-J regions into proximity. Similar mechanisms exist for joining V-D and D-J segments.

There are at least **two additional sources of variety** that contribute to antibody diversity. One of these is a lack of precision in the DNA splicing machinery that fuses V,D and J segments. The site of the junction can vary by several base pairs. Furthermore, in some cases, additional base pairs are inserted in the process of combining segments. Both kinds of change can obviously alter the amino acid sequence of the polypeptide. As a result, even if two antibodies are specified by the same set of gene segments, they may still have slightly different antigen binding sites.

Another major source of diversity is **somatic hypermutation**. Spontaneous genetic changes in the developing cells are known to occur. Mutations are seen in the variable domain genes and in the immediately adjacent regions, but not in the constant domains. Estimates of the rate of mutations suggest that there should be one change in the V region for every three to 30 cell divisions, a rate several orders of magnitude greater than the average mutation rate for eukaryotic genes. It seems likely that B cells or their predecessors carry enzymes that induce mutations in just these sets of genes.

The process of somatic recombination creates a population of cells that vary widely in their antigen specificity; from which few cells are selected by any given antigen. The mutational mechanism is called into action during proliferation of the selected B-cell clones. By altering individual nucleotide bases the mutations “fine-tune” the immune response creating immunoglobulin genes whose products better match the antigen.

The effects of DNA - joining inaccuracy and somatic mutation, in addition, increase the number and variety of antigen binding sites and together with somatic recombination, the total number is estimated at well over a billion different antigen binding types.

Immunoglobulin Class Switching in Individual B-cells, and the Heavy Chain Gene Order

An immature B cell bearing only surface IgM, develops into a cell that simultaneously produces IgM and IgD and is subsequently capable of switching to the production of IgG, IgA or

IgE. It is important to note that each of these heavy chain classes is associated with the same variable heavy chain region (V_H) in a given cell and hence bears the same antigen binding specificity.

The exact mechanism by which the different classes are produced became clearer when the heavy chain gene order was sequenced. As described earlier, this gene order was similar in principle to the V, J and C segments of the light chain gene. As can be seen from Figure 6.4, there are several V_H segments followed by the D_H and J_H segments. The μ constant (C μ) region is closely associated with the δ constant region (C δ). Considerable space separates these two genes from the γ constant (C γ) cluster of genes which appear in the order of Cγ3, Cγ1, Cγ2b and Cγ2a. This is followed by the ε C region and lastly the α C region. The initial rearrangement of the V-D-J segments in the B cell DNA is similar to the light chain genes and has been described earlier. Studies on the various 'C' region segments showed the presence of highly homologous

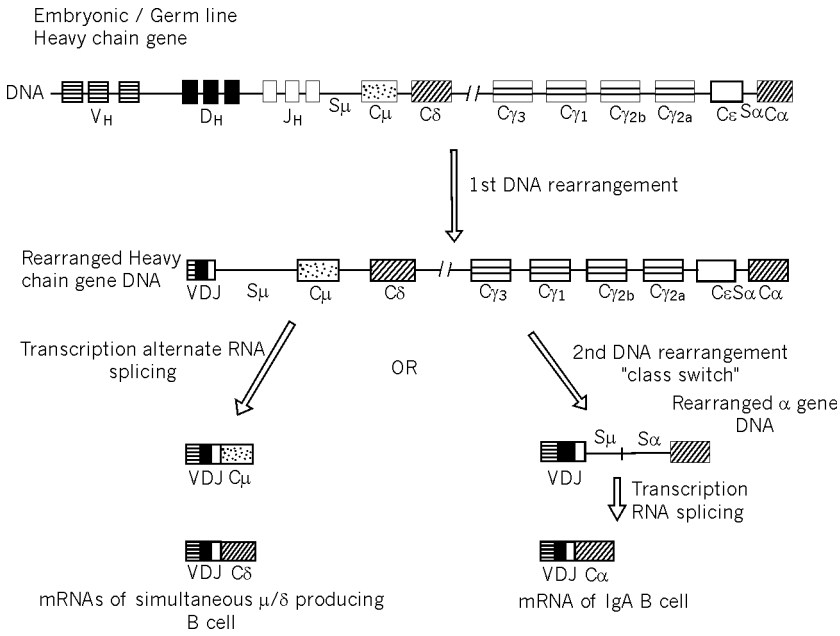


Figure 6.4. Gene assembly for immunoglobulin class switching. Following the initial DNA re arrangement recombining VH, DH and JH region a B cell can utilize alternative sites of RNA splicing to simultaneously produce IgM and IgD. Alternatively such a B cell can further differentiate and switch to producing another class of immunoglobulins. For example a DNA recombination can occur at the switch site (Sα) and bring about a V-D-J-C joining leading to IgA production. Similar homologous switch sites are found (not shown in this figure) in front of each of the 'C' regions.

switch sites (S-sites) in front of the C μ and C α segments (Figure 6.4). Similar switch sites (not shown in diagram) are found in front of each of the other C regions. If RNA splicing occurs at the S μ site, then mRNAs can be produced simultaneously for IgM and IgD by utilizing a transcript with the C μ region for IgM production and the C δ region for IgD production. Such a B cell would be equipped to secrete both IgM and IgD. Alternatively, the same B cell can further differentiate and switch to producing another heavy chain class. This would necessitate a second DNA rearrangement at the highly homologous switch sites of S μ and S α in front of the C μ and C α regions. With transcription and RNA splicing all the intervening sequences are

removed yielding an mRNA consisting of V-V-D-J-C α (see Figure 6.4). Such a B cell would effectively switch to producing IgA with the same antigen specificity. Because similar homologous switch sites are found in front of the γ and ε regions, alternative production of immunoglobulins of all the above classes is possible.

It is essentially correct, however, to state that one B cell produces one kind of antibody, since whatever be the heavy chain class of the immunoglobulin produced, they will all react only to one antigenic determinant, since they all have similar variable regions and therefore similar antigen binding specificity.

CHAPTER – 7

THE COMPLEMENT SYSTEM

The complement system is the primary humoral mediator of antigen antibody reactions. The system consists of at least 20 chemically and immunologically distinct plasma proteins, capable of interacting with each other, with antibody and with cell membranes. Following activation of this system, these interactions lead to the generation of a wide range of biological activity from lysis of different kinds of cells, bacteria and viruses to direct mediation of the inflammatory process. In addition, complement is able to recruit and enlist the participation of other humoral and cellular effector systems and induce histamine release from mast cells, migration of leucocytes, phagocytosis and release of lysosomes from phagocytes.

The individual proteins of the complement system are normally found in the serum as functionally inactive molecules, belonging to the plasma globulin fraction. Components are designated by numerals C_1, C_2, C_3 etc and by names such as Factor B and Factor D. Each component is activated sequentially (similar to the triggered enzyme cascade phenomenon in coagulation), resulting in the complement reaction. Once activated the complement components become enzymes and are designated with a bar such as $\overline{C1}$ or $\overline{\text{Factor B}}$. A complement component can sometimes be cleaved by an enzyme leading to the formation of cleavage products such as C4a and C4b.

There are two parallel but entirely independent pathways leading to the terminal most active part of the complement system (Figure 7.1). The two pathways are called the **classical** and the **alternative** pathways. Each pathway is triggered by different substances. The two pathways converge at a point from which the **final common pathway** ensues, to terminate at the end point of complement activation which is **cytolysis** or **cytotoxicity**. There are other non complement enzymes of serum or cellular origin which can activate the complement system midway for example : trypsin - like enzymes such as plasmin.

Table 7.1: Activators of the complement system

	Classical	***Alternative***
Immunologic	Antigen-antibody complexes	IgA, IgG
Non-immunologic	Trypsin-like enzymes DNA C-reactive protein Staphylococcal protein A.	Lipopolysaccharide, plant and bacterial polysaccharides, cobra venom; Trypsin-like enzymes,

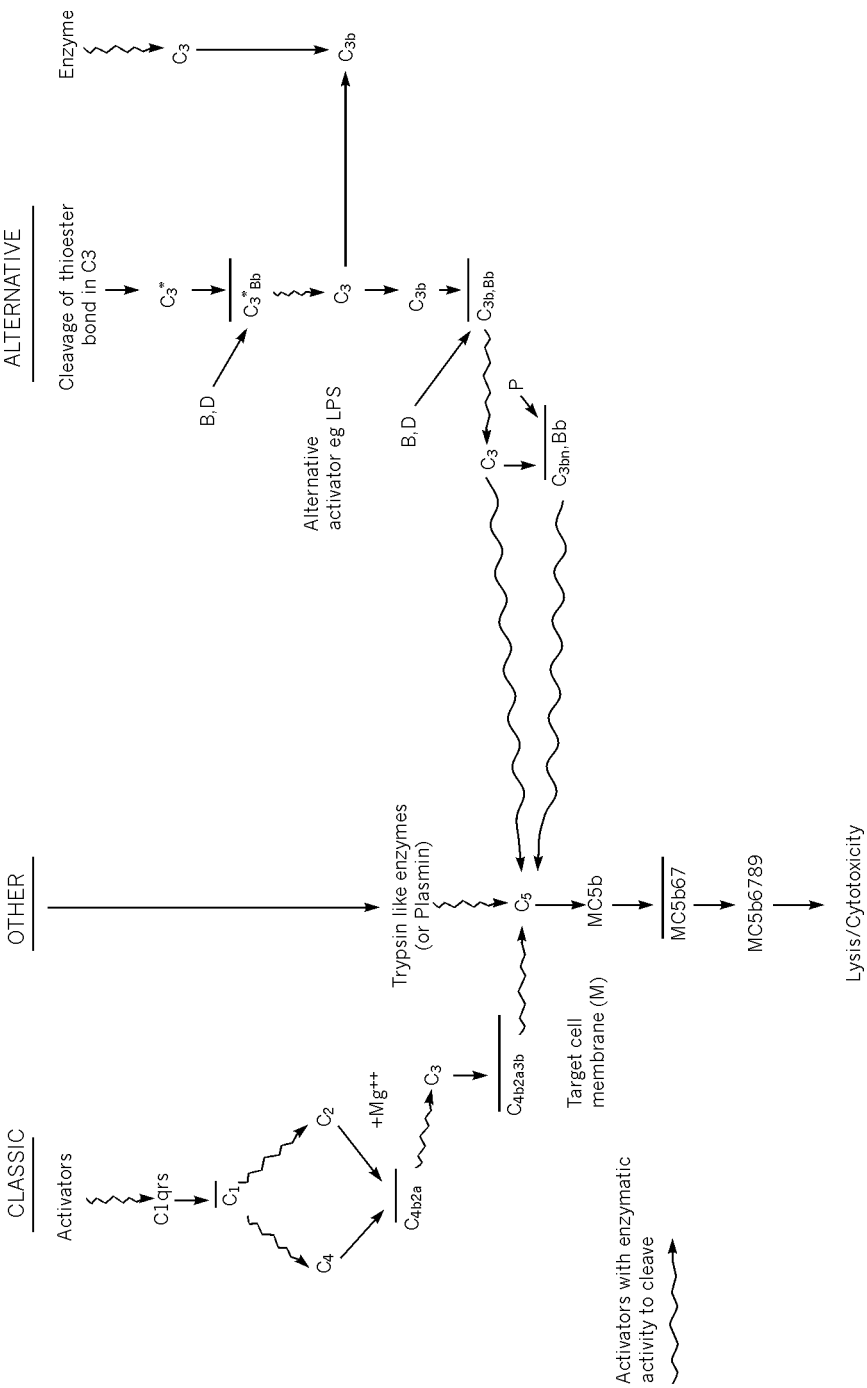


Figure 7.1. The Complement Cascade.

The Classical Complement Pathway

The classical complement pathway is activated as described above (refer Table 7.1). Among the IgG sub classes, IgG_3 is the most active, followed by IgG_1 and IgG_2. IgM is the most efficient immunoglobulin activator of the classical complement pathway. Activation occurs by binding of the first complement component C1, to a site located in the CH_2 region of the immunoglobulin molecule. Hence antigen antibody complexes or aggregated immunoglobulins are a must for activation of the classical complement system. As listed, non immunologic substances also activate complement. Here, activation occurs by direct binding of C1 or in the case of enzymes, by direct proteolytic attack of C1.

C1

The steps involved in the activation of C1, following attachment or proteolytic attack, comprise the first functional unit of the complement pathway. C1 consists of 3 distinct protein molecules: C1q, C1r and C1s held together by calcium bonds. C1 is normally present as a complete entity; individual proteins of C1 are found only in disease states. C1q is unique because its structure is very similar, chemically, to that of collagen or basement membrane.

C1q contains a total of 18 polypeptide chains of 3 distinct types, that are organized into a structure consisting of 6 peripheral globular portions connected by fibrillar strands to a central structure. The polypeptide chains have been visualized as forming 6 sub units, each in the form of a triple helix (Figure 7.2).

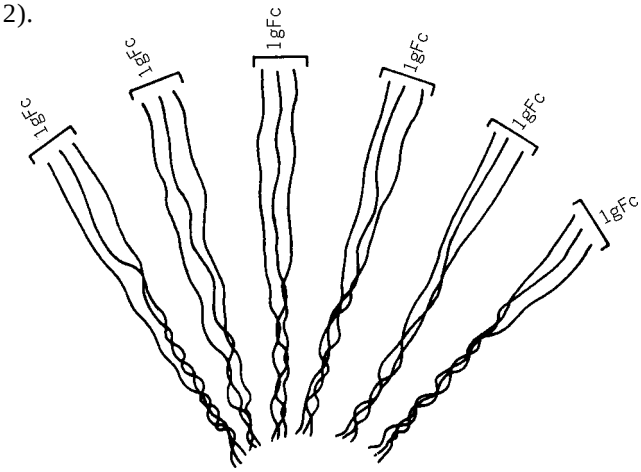


Figure 7.2. Schematic representation of C1q.

The C1q molecule bears the sites which enable the C1 molecule to bind to the Fc region of IgG and IgM antibodies. It is therefore able to bind to 6 IgG molecules (Figure 7.2).

Once C1q binds to the immunoglobulin molecule, C1r, a β globulin molecule, acquires the ability to enzymatically activate C1s.Integrity of the C1 macromolecule and calcium ions are required for this process. C1s, once activated, $(\overline{C1s})$ acquires proteolytic activity. Once C1s is activated the initial phase of the classical complement pathway is completed; the earlier reactants including antigen, antibody and C1q or C1r are not necessary for progression of the complement reaction. Activated C1s mediates the next phase of the complement cascade.

C4 and C2

C1s cleaves C4, yielding C4a and C4b (Fig 7.3); it also cleaves C2 and initiates the formation of the key enzyme $\overline{C4b2a}$ on the activator surface. C4b contains a labile binding site which

binds to its activator in a transient manner. C2a also has a labile binding site which allows it to bind to C4b . Magnesium ions are required for the formation of the $\overline{C4b2a}$ complex. Activated $\overline{C4b2a}$ is a proteolytic enzyme that assumes the role of continuing the classical complement reaction. Once it has been formed, earlier reactants are no longer necessary. C4b2a is also termed C3 convertase as it cleaves and thereby activates C3. The enzymatic site resides in the C2 moiety of the complex.

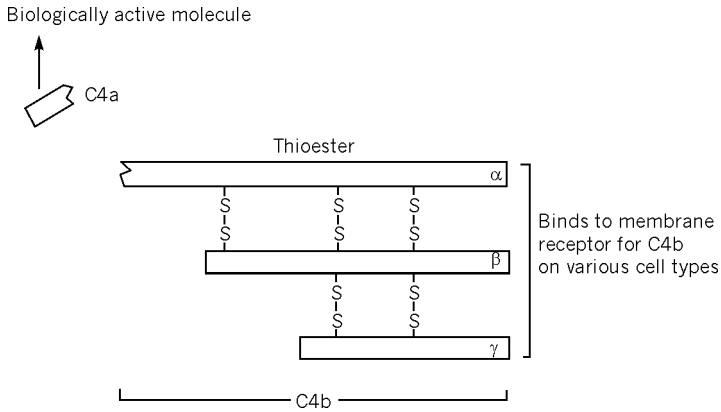


Figure 7.3. Schematic model of C4: disulphide linkages between the three chains go into forming the C4 molecule.

C3

Cleavage of C3 results in the formation of C3a and C3b (Figure 7.4). C3a is a biologically potent peptide and will be discussed later. The attachment of C3b to membranes in the vicinity of $\overline{C4b2a}$ molecules leads to the generation of the last enzyme of the classical pathway: $\overline{C4b2a3b}$. This enzyme acts to cleave C5.

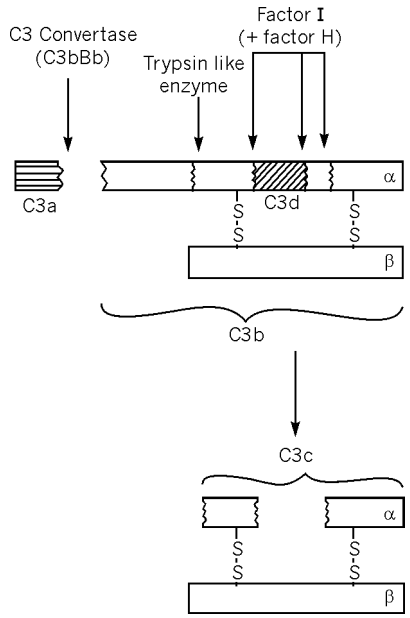


Figure 7.4. Schematic structure of C3 and its cleavage products. The B chain together with the disulphide linked fragments remaining after Factor I cleavage is termed C3c.

The classical complement pathway thus consists of a series of enzyme substrate reactions in sequence, which lead to the formation of several more complement enzymes. The reactions involved are highly specific and there is considerable turnover of the molecules involved i.e. C2, C3, C4 and C5. In addition, several biologically active molecules are generated and it is evident that a relatively small stimulus to complement activation may lead to considerable generation of these molecules with the result that severe tissue reaction can ensue.

The Alternative Complement Pathway

The alternative complement pathway constitutes the humoral component of natural defence against infections which can operate without antibody participation. Six proteins, C3, B, D, H, I, and P, by themselves perform the functions of initiation, recognition, and amplification of the pathway. A variety of activators of this pathway have been described such as certain particulate polysaccharides, for example, bacterial (LPS), yeast (zymosan), or plant (inulin) polysaccharides, fungi, bacteria, viruses, certain mammalian cells, and aggregates of immunoglobulins, for example, the Fab portions of IgA or IgE (Table: 7.1).

It is not yet known which structures these activators have in common as far as recognition by the pathway is concerned. The mechanisms by which these heterogeneous groups of substances can initiate the activation of this pathway, are also not fully understood. It is clear, however, that recognition involves C3b.

Proteins of the alternative complement pathway

- P (properdin) – a γ2 globulin
- factor B – a β2 protein
- factor D – an α globulin
- factors I and H – β globulins

The alternative complement pathway, because of its major role in the resistance to infection, is considered to be the pathway that is more important to man. The alternative pathway was so named because activation proceeds in a manner different from that of the classical pathway, and bypasses the need for C1, C4 and C2.

An initial requirement for activation is the presence of C3b, which is continuously generated. This occurs following **cleavage of a thio ester bond in C3**, thus forming **C3***, which reacts with factors B and D to generate an enzyme able to cleave C3 into C3a and C3b (the C-3 cleaving enzyme, also known as the priming C3 convertase). Water can hydrolize C3 and form C3i, a molecule that functions in a manner similar to C3b. It is possible that C3 in circulation is also cleaved by enzymes of the coagulation and fibrinolytic systems; the enzyme zymosan : a yeast polysaccharide also causes C3 activation in the presence of magnesium ions. Most of the newly generated C3b remains in the fluid phase, some binds to various cellular surfaces. In either case this C3b is rapidly inactivated by control proteins, factors I and H, which cleave it. This steady level of C3b in the body is greatly modified by particulate activators of the alternative pathway, such as lipopolysaccharides and certain cells. The C3b deposited on such activators is “protected” from destruction by factors I and H. This surface bound, protected C3b interacts with factor B in the presence of magnesium ions to form C3bB. With the addition of factor D, which acts on bound B to cleave it yielding Bb, an enzyme $\overline{\text{C3bBb}}$ is generated. This surface bound enzyme is also called the amplifying C3 convertase and is able to cleave very large amounts of C3. This results in the accumulation of C3b and as shown in Figure 7.5, there is a cyclical amplification of the critical C3b component. This is also called a positive

feed back mechanism which enhances C3b formation. Furthermore, if properdin (P) were to complex with $\overline{C3bBb}$ yielding $\overline{C3bPBb}$, the enzyme becomes functionally more efficient.

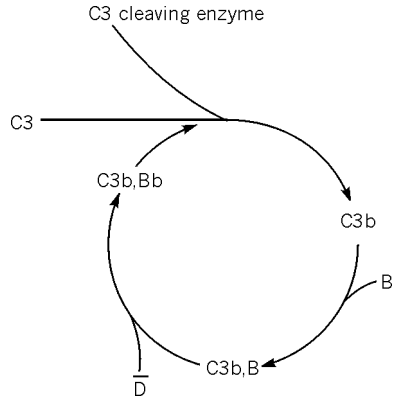


Figure 7.5. The positive feed back mechanism for C3b.

Many of the C3b molecules thus generated, either by $\overline{C3bBb}$ or $\overline{C3bPBb}$, bind to the surface of the activator particle in close proximity to each other. This results in the formation of modified enzymes, $\overline{C3b_nBb}$ or $\overline{C3b_nPBb}(n>1)$, which are able to cleave C5 and initiate the final common membrane attack pathway (see Figure 7.1). Hence the cyclic amplifying system, in conjunction with the crucial "protected" surface represents the key events in activation of the alternative pathway.

Cell surface sialic acid, which is present in glycoproteins, glycolipids and polysaccharides increases the affinity of membrane associated C3b for H. Thus cells that have abundant sialic acid are non activators of the alternative pathway. It has been hypothesized that some bacterial species that carry capsular sialic acid are more pathogenic for neonates than organisms that lack this substance. Bacteria with capsular sialic acid include type III group B *Streptococcus*, groups B and C *Neisseria meningitidis* and K1 *Escherichia coli*. Human parasites such as *Leishmania major* and *Trypanosoma cruzi* have evolved several strategies for evading recognition by the alternative pathway, including synthesis of glycoproteins that contain sialic acid, rapid degradation of bound C3b and synthesis of a protein that competes with B for binding to C3b.

Host cells are protected from the effects of complement activation by membrane proteins which prevent activation of classical and alternative pathways. The alternative pathway may be activated by an isolated protein obtained from cobra venom. This protein appears to represent cobra C3b.

The Membrane Attack Mechanism: C5 - C9

The terminal portion of the complement sequence is termed the membrane attack system, since C5b-9 must become membrane bound in order for membrane changes or damage to occur.

The complement attack mechanism is initiated on cleavage of C5 by $\overline{C4b2a3b}$, $\overline{C3b_nBb}$ or $\overline{C3b_nPBb}$ or certain enzymes such as plasmin (see Figure 7.1). The activation reaction results

in generation of a biologically active peptide: C5a and a larger C5b. C5b has the ability to bind C6 and C7 forming a complex of $\overline{C5b67}$. This complex has only transient ability to bind to membranes. However, this process is modulated by S protein which is a normal serum protein. The S protein acts by binding to the membrane at the same binding site as the $\overline{C5b67}$ complex, thus naturally inhibiting it. Each $\overline{C5b67}$ complex possesses a binding site for the molecule C8. Membrane leakage begins at this stage. The cytolytic process is,however,greatly enhanced by the attachment of the last complement component: C9, to the membrane bound $\overline{C5b678}$ complex. The resulting C5b6789 complex, containing one molecule of C5b,C6,C7,C8 and several molecules of C9, represents the fully assembled cytolytic mechanism of the complement system.

The Molecular Mechanism of Cytolysis

Binding of antibody to a target cell triggers the complement cascade in which successive proteins of the complement system are activated as described in the previous section. Eventually C5b binds to C6 and C7 and is inserted into the target cell membrane. C8 and several C9 proteins then aggregate around the C5b67 complex binding together to form a cylindrical channel or a pore like structure. This permits rapid influx of ions because of a leaky membrane and ultimately leads to cell lysis (Figure 7.6). Just one such membrane - attack complex (or pore channel) of the complement system need be inserted into the target cell to cause cell lysis. This is called the “one-hit” mechanism of complement mediated cell lysis.

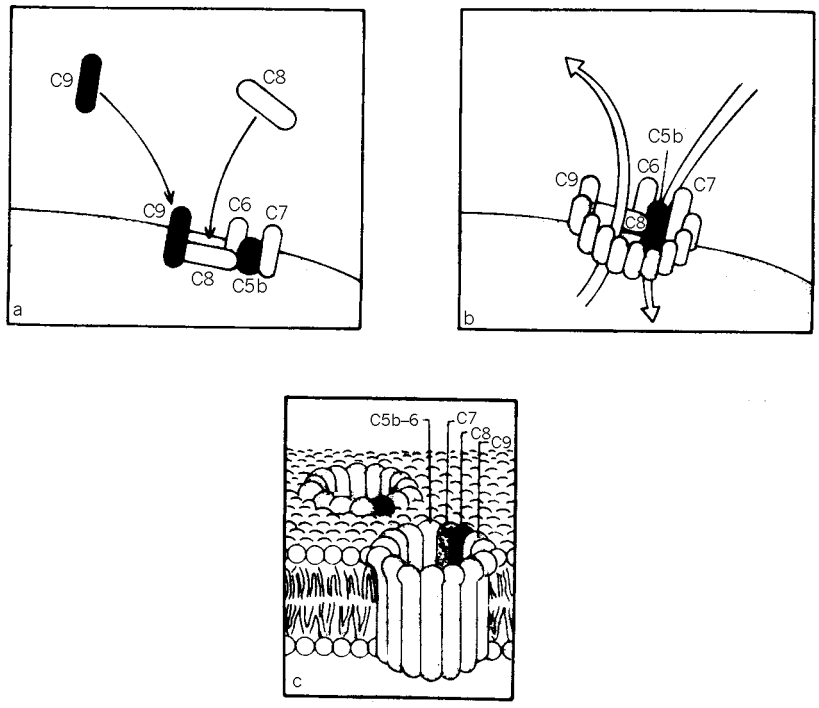


Figure 7.6. The mechanism of complement mediated membrane leakage.

Control Mechanisms of the Complement System

Uncontrolled activation of the complement system is prevented by the fact that active sites of the complement components are labile. In addition, several complexes such as $\overline{C3bBb}$, $\overline{C4b2a}$ and $\overline{C4b2a3b}$ dissociate in a time and temperature dependant fashion.

Other proteins limit runaway complement activation by binding to or enzymatically attacking certain specific complement components. **C1 inhibitor** is one such protein which inhibits enzymatic activity of C1 and its C1r and C1s subunits by rapidly forming firm, irreversible complexes.

Another key control protein of the complement system is **Factor I**. Factor I attacks C3b free, in solution or on the surface of cells and cleaves the molecule. Another regulator that acts on C3b is **Factor H**, a serum protein that binds to C3b and accelerates the destruction of C3b by factor I. It also binds to C3b on intermediate complexes and inhibits the enzyme $\overline{C3bPBb}$. A **C4 binding protein** also exists which binds to C4b and facilitates its destruction by Factor I. A **C8 binding protein** inhibits binding of C9 to $\overline{C5b678}$ and prevents the membrane attack mechanism from going into operation. Human serum contains an enzyme the **anaphylatoxin** inactivator, an α globulin, which destroys the biological activities of C3a, C4a and C5a. The S protein binds to the $\overline{C5b67}$ complex and prevents membrane leakage.

Biological Consequences of Complement Activation

Besides the labile binding sites involved in the pathways, sites are generated or uncovered in the fragments of C3 and C4 as a consequence of proteolytic cleavage of the molecules during complement activation. Proteolytic cleavage also results in further breakdown products of the molecules C3 and C4. There are therefore several reactive sites generated in C3b, C4b and in their breakdown fragments. These reactive sites are recognised by various cells having specific receptors for these fragments. Table 7.2 lists the various complement fragments, their receptor specificity and the cells bearing these receptors.

TABLE 7.2: SPECIFICITY AND CELLULAR DISTRIBUTION OF COMPLEMENT RECEPTORS

Receptor	*Complement fragment*	*Cell type*
CR1	C3b	Erythrocytes,PMNs; B-lymphocytes; subsets of T-lymphocytes; monocytes; macrophages; dendritic cells
CR2	C3d	B-lymphocytes
CR3	C3bi	PMNs, monocytes, macrophages
CR4	C3d	PMNs, monocytes
C3a and C5a	C3a, 4a, 5a	PMNs, monocytes, macrophages, mast cells

Results of Complement–Receptor Interactions

The major complement receptors are:

(*i*) CR1 preferentially binds the initial C3 activation product C3b. These receptors are found on many cell types as listed in Table 7.3. The activator particle bearing the antibody-C3b complex is therefore attached to various cell types by these CR1 receptor molecules. The immune complex forms a bridge between the activator and the cell type. When the cell type involved is the erythrocyte (see Figure 7.7), there is an immune red cell adherence around the activator-antibody-C3b complex. This helps to regulate complement activation and aids in the clearance of immune complexes, as red cells "carry" these complexes to the liver for degradation.

On certain lymphoid cells and phagocytic cells the receptors help to link the activator particle via the antibody-C3b link to phagocytic cells. This helps to augment phagocytic destruction of an activator particle that is coated (opsonized) by the antibody- C3b complex (Figure 7.7).

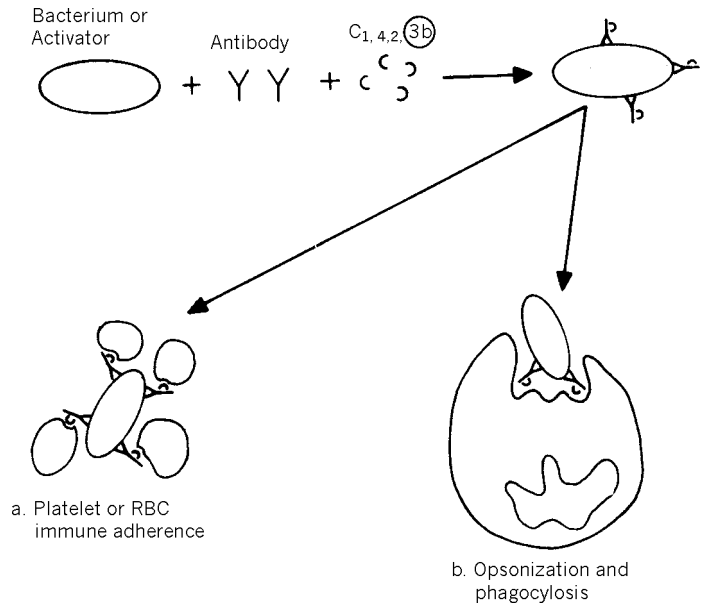


Figure 7.7. Biologic consequences of complement-receptor interactions

(*ii*) The receptor known as CR2, binds the terminal C3 cleavage products i.e. C3d.This receptor is confined to B lymphocytes. CR2 has also been shown to be the structure used by the Epstein-Barr virus to attach to and infect B cells. CR2 plays an important role in the presentation of antigen to specific B and T cells and in the control of B-cell proliferation. Evidence clearly suggests that CR2 is involved in the induction of a primary humoral response.

(*iii*) The CR3 receptor binds the intermediate complement C3 cleavage product, C3bi. Interactions with C3bi and receptors on phagolytic cells enhance antibody dependant phagocytic responses, leading to destruction of the activator. Individuals genetically lacking CR3 are predisposed to recurrent life threatening bacterial infections.

(*iv*) CR4 is a C3 receptor that binds C3bi and C3d. CR4 has an important role in host resistance to infection. C3bi-coated immune complexes have a high affinity for the

CR4 receptors on phagocytic cells of the liver and spleen, to where they are transported and degraded.

(*v*) Receptors for C3a and C5a bind to anaphylatoxins (see below).

Biologic Actions of Complement Cleavage Products

The low molecular weight fragments of C3, C4 and C5 - C3a, C4a and C5a respectively are known as anaphylatoxins. These hormone like peptides induce smooth muscle contraction, enhance vascular permeability, release vaso active amines from mast cells and basophils and induce lysosomal enzyme release from granulocytes.

In addition, C5a is also chemotactic ie: it is able to induce the migration of leukocytes into an area of complement activation. The C5a molecule has a number of other properties, which include granulocyte aggregation, and activation of intracellular processes in certain cells, leading to various effects such as release of oxygen metabolites and SRS-A (slow reacting substance of anaphylaxis). Histamine induced effects of C3a, C4a and C5a are blocked by anti histamines.

There are other biologic consequences of the complement activation system. These include the generation of a kinin, possibly a fragment of C2, which causes smooth muscle contraction and increased vascular permeability. This kinin does not function through release of histamine.

Biologic Significance of the Complement System

The sum total effect of the integrated complement system is that it is able to produce inflammation and facilitate the localization of an infective agent. (Figure 7.8). The C3b, C4b receptors on phagocytic cells help link phagocytic cells to activator particles that are coated with antibody and C3b or C3b alone. This coating and facilitation of phagocytosis has been called opsonization. The term opsonin is derived from the greek word “opsons” meaning to prepare food for: (phagocytes).

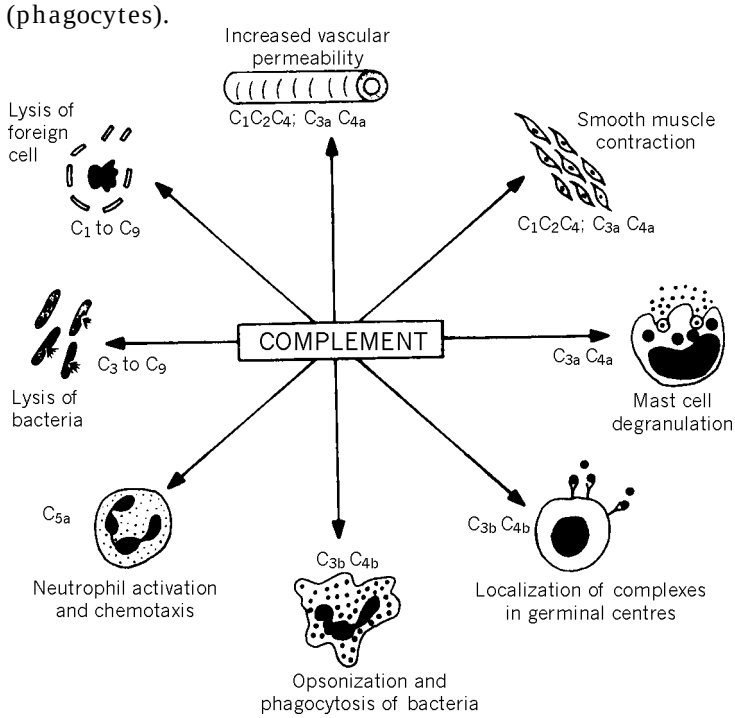


Figure 7.8. Biological effects of complement mediated reactions

A classic description of opsonin was given in 1906, by George Bernard Shaw, in his play "The Doctors Dilemma". The lead character Sir Colenso Ridgeon was closely patterned after the British scientist Almroth Wright. "The phagocytes" Ridgeon says in the play, "won't eat the microbes unless the microbes are nicely buttered for them. Well the patient manufactures the butter for himself alright, that butter, I call opsonin ." There are two principal classes of opsonin: specific serum antibody and complement component fragments.

Other biologic effects such as smooth muscle contraction, vascular permeability and chemotaxis of leukocytes facilitate the acute inflammatory process.

Evidence for the biologic importance of this system can be obtained from human and animal complement deficiency states. There is a marked increase in susceptibility to infection. Infections and other human disorders associated with congenital complement deficiencies are given in Table 7.3.

Table 7.3: Human disorders associated with congenital complement deficiencies

C1q	Systemic lupus erythematosus(SLE) or similar syndrome, hypogammaglobulinaemia, nephritis
C1r	Renal disease, SLE or similar syndrome, recurrent infections, rheumatoid disease
C1s & C4	SLE
C2	Arthralgia, SLE or similar syndrome, nephritis, susceptibility to infection
C3	Recurrent infections with pyogenic bacteria
C5	SLE, recurrent infections, recurrent gonococccal infections
C6	Recurrent gonococcal and meningococcal infections and Raynaud's phenomenon
C7	Recurrent gonococccal and meningococcal infections, SLE
C1 inhibitor	Hereditary angioedema
C3b inhibitor	Repeated infections, recurrent infections with pyogenic bacteria

CHAPTER – 8

DETECTION AND APPLICATION OF ANTIGEN–ANTIBODY REACTIONS

Basic knowledge regarding the molecular basis of antigen and antibody structure and function, will eventually influence diagnosis and therapeutics in clinical medicine. Much of this chapter will be devoted to the dynamics and clinical laboratory applications of antigen-antibody interactions.

For ease of description these antigen antibody reactions have been broadly classified into:

- Precipitation
- Agglutination
- Complement fixation
- Immuno assay using labelled reagents
- Immuno histochemistry (Immunofluorescence)
- Cytokine immunoassays (ELISPOTR)
- DNA innunoassays

Precipitation

The classical precipitation reaction was first demonstrated by **immunodiffusion** in gel. It detects the reaction between **soluble antigen** and a potent antiserum mixed in the correct proportions. The antigen-antibody precipitate that is so formed in any semi solid medium such as agar or agarose is also dependent on buffer electrolytes, pH and temperature. The formation of precipitation lines in any immunodiffusion system is highly dependent on relative concentrations of antigen and antibody.

This relationship is depicted schematically in Figure 8.1, maximal precipitation is formed in the zone of equivalence; not in the zones of antigen or antibody excess. The **prozone phenomenon** (Figure 8.2), is a term which is used to describe suboptimal precipitation or incomplete lattice formation which occurs in the region of antibody excess. A similar phenomenon occurs in the region of antigen excess and is sometimes called the post zone effect.

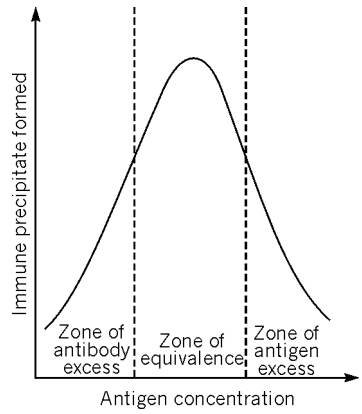


Figure 8.1. The zone of equivalence in antigen antibody reactions: where maximal precipitate is formed.

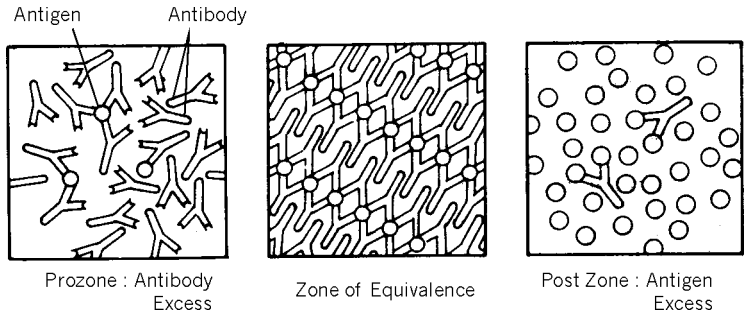


Figure 8.2. The optimal ratio of antigen to antibody (middle) yields an insoluble precipitate in the classic precipitation test. Extreme antibody excess (left) or antigen excess (right), however, does not lead to lattice formation.

Immunodiffusion or Agar Gel Diffusion

Agar gel diffusion is the simplest and most direct means of demonstrating soluble antigen-antibody reactions in the laboratory. In 1946 **Oudin** described a system of single diffusion of antigen and antibody in agar filled tubes. This important advance was soon followed by **Ouchterlony's** classic description of double diffusion in agar layered on slides. This method is still widely used today to detect and analyze antigen-antibody reactions.

Agar gel diffusion reactions may be classified as single or double. In single agar gel diffusion either antigen or antibody remains fixed and the other reactant is allowed to diffuse freely in the gel. In double immuno diffusion both reactants are free to move towards each other and precipitate. Movement within the gel may be linear or radial. The most important clinical application of immunodiffusion is the quantification of serum immunoglobulins.

(*i*) *Ouchterlony's Double Diffusion*

This test is performed by pouring molten agar onto glass slides or into Petri dishes and allowing it to harden. Small wells are punched out of the agar, a few millimetres apart. Samples containing antigen and antibody are placed in opposing wells and allowed to diffuse toward one another in a moist chamber for 18- 24 hours. Antigen and antibody diffuse out of the wells in a radial fashion. Two arcs from each well approximate with each other to form a line of precipitation, which can be viewed by indirect light or with a magnifying lens (Figure 8.3). Gels can be dehydrated and stained by protein binding dyes like Coomassie brilliant blue and preserved indefinitely.

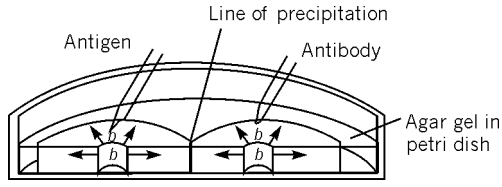


Figure 8.3. Agar-gel diffusion.

Double diffusion can also be performed between one or more antigens and a single antibody for comparative purposes. The three basic characteristic patterns of such reactions are shown in Figure 8.4. Double diffusion in agar can also be used for semi-quantification of antigen or antibody reactants as shown in Figure 8.5. It is still an important initial test for the detection of serologic reactions to various human, animal and plant antigens.

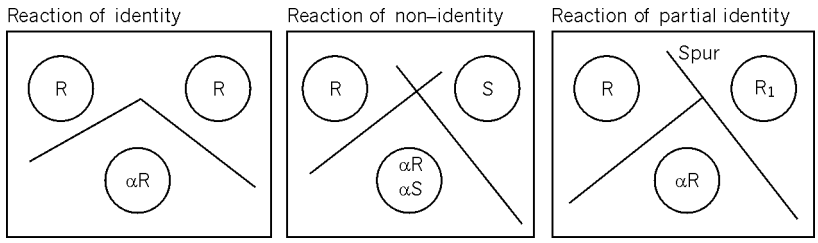


Figure 8.4. Reaction patterns with double diffusion in agar R=antigen-R, S=antigen-S, R1=antigen-R1, αR = antibody to R; αS = antibody to S

Reaction of identity : Precisely similar precipitin lines have formed in the reaction between R and anti R. The lines meet at a point, showing that the two antigens are similar. Reaction of non-identity. Precipitin lines completely cross owing to two separate interactions: R with αR and S with αS; showing that R and S are dissimilar and non cross- reacting antigens. Reaction of partial identity: αR reacts with both R and R1; the precipitin lines do not cross, a characteristic spur is formed, indicating that antigenic determinants are partially shared R and R1.

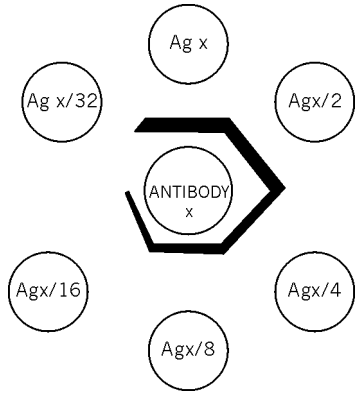


Figure 8.5. Semi-quantitative analysis using double immunodiffusion; semiquantitative analysis of antigen. Antigen x is serially diluted and placed in a ring of wells surrounding a central well containing antibody to x. Precipitin lines form with decreasing thickness until no longer visible at an antigen dilution of 1:32. The whole process can be reversed to quantitate antibody.

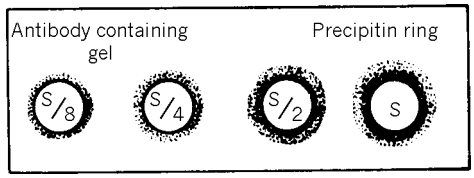


Figure 8.6. Single radial immuno diffusion. Antibody is added to the agar which is then poured onto a slide or petri dish. Wells are punched in the agar and dilutions of test antigen-S are put in the wells. The plates are left for 24 hours during which time antigen diffuses out of the wells and binds to antibody; when the point of equivalence is reached, antigen and antibody precipitate to form a ring. The area within the precipitin ring is proportional to the antigen concentration.

(*ii*) *Single radial immunodiffusion*

The double immunodiffusion is only semi quantitative. In 1965, Mancini introduced a single diffusion technique for quantitative estimation of antigens. Radial diffusion is based on the principle that a quantitative relationship exists between the amount of antigen placed in a well (where the antibody is incorporated into the gel) and the resultant ring of precipitation (Figure 8.6). An important application of this test is in the detection and semi-quantification of many antigens.

Electrophoresis

Simple zone electrophoresis uses the principle of separation of individual proteins (in a complex protein mixture such as human serum) in an electrical field.

The medium in which this occurs is generally one that does not impede or enhance the flow of molecules in an electrical field. Generally, paper, agarose or cellulose acetate strips are used for this purpose. Technically, the biologic fluid is spotted at the origin and subjected to an electrical current, allowing for separation of individual constituents. Large molecular weight substances move slowly and traverse a shorter distance in the given time period, smaller molecular weight fragments traverse further. Densitometer scanning converts the separated bands into peaks. Figure 8.7, shows separation of normal human serum into 5 major electrophoretic bands: albumin, $\alpha 1$ globulin, α 2 globulin, β globulin and γ globulin. Human immunoglobulins fall into the γ globulin fraction of human serum.

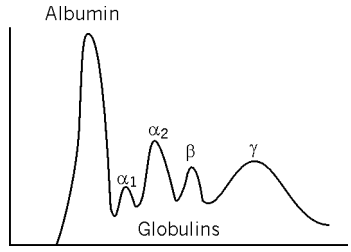


Figure 8.7. Electrophoretic separation of normal human serum.

Zone electrophoresis is extremely valuable in the diagnosis of human paraprotein disorders and disorders of the γ globulin fraction of serum such as hypo or agammaglobulinemia.

Immunoelectrophoresis (IEP)

Immunoelectrophoresis combines electrophoretic separation and immunoprecipitation of proteins. Both identification and approximate quantification can thereby be accomplished for individual proteins present in serum, urine or other biologic fluids. The technique is illustrated in Figure 8.8, using human serum as antigen and antiserum raised against whole human serum as antibody. The applications of immunoelectrophoresis include diagnosis of paraproteinemias, hypo or agammaglobulinemia and identification of L chains in the urine of patients with plasma cell dyscrasias or autoimmune disorders. Thus with specific anti-κ or anti-λ antisera, the monoclonal nature of Bence-Jones proteins in myeloma can be confirmed. Immunoelectrophoresis is also helpful in identifying increased amounts of proteins present in the cerebro spinal fluid in patients with various neurologic diseases.

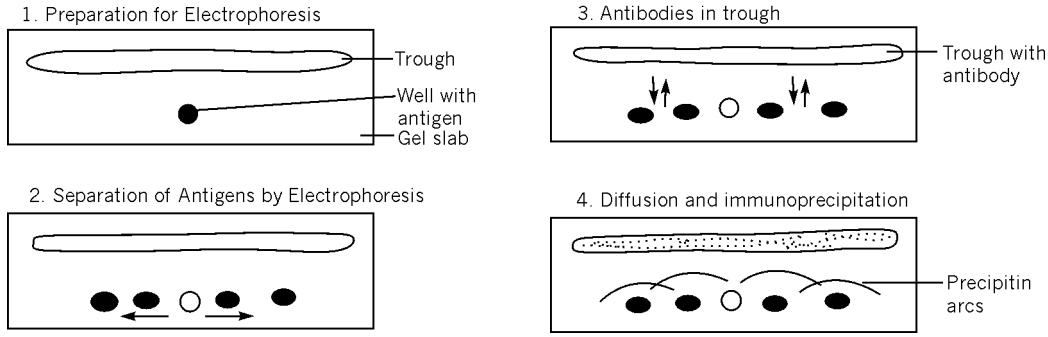


Figure 8.8. Technique of immunoelectrophoresis : (1) Agar gel slab is prepared, a trough and well are cut and the well is filled with antigen. (2) Antigens are separated by electrophoresis and the pH of the gel is chosen so that positively charged proteins move to the negative electrode and negatively charged proteins move to the positive electrode. (3) After antigen separation, the trough is filled with antiserum which is left to diffuse. (4) The separated antigens and the antibodies in the trough diffuse, interact and form precipitin arcs. Immunoelectrophoresis permits the comparison of complicated mixtures of antigens found in human serum.

Electroimmunodiffusion

In imm unodiffusion techniques described this far, antigen and antibody are allowed to come into contact and to precipitate in agar purely by diffusion. However, the chance of an antigen and antibody meeting and the speed of development of a precipitation line can be greatly enhanced by electrically driving the two together. Two variations of electroimmunodiffusion have received clinical applicability. They are (*i*) Counter immunoelectrophoresis (CIE) and (*ii*) Laurell's rocket electrophoresis.

(*i*) *Counter immunoelectrophoresis*

The basic principle involves, as before, pouring molten agar on a slide and punching wells into it a few millimetres apart, once the gel has solidified. Antigen and antibody are placed in the opposing wells. In a clinical condition the biologic fluid is placed in one well and known antibody in the other. This detects antigen in the clinical material. The system can be reversed to detect antibody in the clinical sample. The slide is then placed in an electrophoresis tank and bathed in buffer solutions of optimal pH and connected to a power pack supplying the electrical current. As shown in Figure 8.9, the antigen solution being a protein moves towards the positive pole. The proteins in the given antiserum well also move towards the positive pole. However, the γ globulin fraction which contains all the immunoglobulins is driven in the opposite direction by electro endosmosis. This drives antibody towards the antigen and precipitin lines may be seen to be formed a few hours after beginning of electrophoresis. Electro endosmosis is a phenomenon in which molecules within the agar gel tend to move in the direction opposite to the flow of current when in an electrical field. γ globulins being relatively low molecular weight substances are hence carried along by electro endosmosis towards the antigen, facilitating the antigen- antibody reaction.

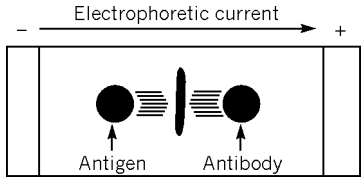


Figure 8.9. Counter immuno electrophoresis. Antigen and antibody are placed in wells and driven towards each other by electric current, a precipitin line forms in the zone of equivalence, where antigen and antibody meet.

This technique can produce visible precipitin lines within 30 minutes and is 10 times more sensitive than agar gel diffusion. However, it is only semi quantitative. CIE is widely used in many plant, animal and human immunology laboratories for the detection of normal and abnormal proteins.

(*ii*) *Laurell's rocket electrophoresis*

In this technique antiserum to the particular antigen or antigens is incorporated into an agarose medium and poured over a glass slide. This ensures that antibody does not migrate. The specimen containing the unknown antigen is placed in a small well. Electrophoresis of the antigen into the antibody containing agarose is then performed. The resultant pattern of immuno-precipitation resembles a spike or rocket – hence the term rocket electrophoresis (Figure 8.10).

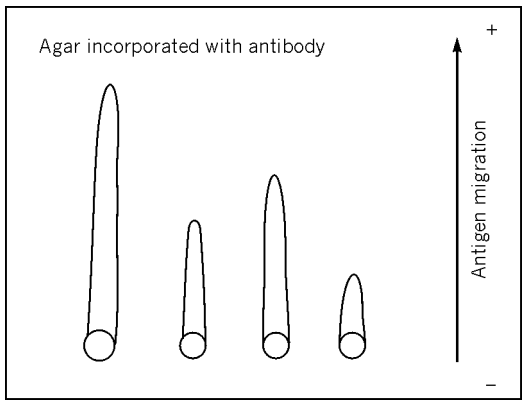


Figure 8.10. Rocket electrophoresis (Laurells' technique). Varying concentrations of the antigen are placed in wells 1-4. Electrophoresis is performed and antigen is driven into the antibody - containing gel. Precipitin pattern forms in the shape of a "rocket." Amount of antigen is directly proportional to height of the rocket.

The principal application of this technique is to quantify antigens in biological fluids. The rocket pattern occurs because precipitation occurs along the lateral margins of the moving boundary of antigen, as the antigen is driven into the agar containing the antibody. Gradually as the antigen is lost through precipitation its concentration at the leading edge diminishes and the lateral margins converge to form a sharp point. The total distance of antigen migration (length of spike) for a given antiserum concentration is linearly proportionate to the antigen concentration. This system cannot be used to quantify immunoglobulins because their weak negative charge prevents their electrophoretic mobility in this system.

(*iii*) *Crossed immunoelectrophoresis*

One powerful variant of Laurell's rocket system, the crossed immunoelectrophoresis involves a preliminary electrophoretic separation of an antigen mixture in a direction perpendicular to that of the final "rocket stage". In this way each of several antigens in a mixture can be quantified (Figure 8.11). The technique is used in many immunology laboratories for the detection of antigens in human and animal biological fluids.

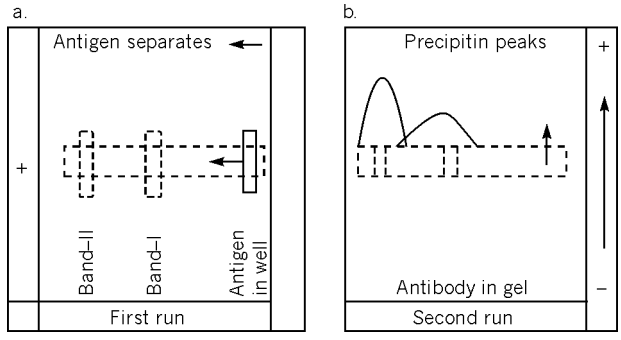


Figure 8.11. (a) Antigens are separated on the basis of electrophoretic mobility in agar gel (dotted vertical bands). (b) A narrow longitudinal strip (dashed lines) containing the separated antigens is cut out as shown, laid over another gel, which contains antibody and electrophoresis carried out in a direction at right angles to the first run to drive the antigen bands through the antibody containing gel. Antigen and antibody complex to form precipitin peaks. The area under the peak is related to the concentration of antigen.

Agglutination

Whereas cross linking of multivalent protein antigens of a soluble nature leads to precipitation, cross-linking of cells or large particles by antibody directed against surface antigens leads to agglutination. Since most cells are electrically charged, a reasonable number of antibody links between two cells is required before the mutual repulsion is overcome. The agglutination of cells bearing only a small number of surface determinants may therefore be difficult to achieve. Similarly the higher avidity of multivalent IgM antibody relative to IgG, makes it a far more effective agglutinating agent. Important advantages of agglutination are that reactions can be read visually, have a high degree of sensitivity and an enormous variety of substances are detectable through this method.

Agglutination reactions may be classified as either direct or indirect (passive). Besides these there are several modifications of the agglutination principle, which have been used successfully in clinical, laboratory medicine.

Agglutination techniques

(*i*) *Direct agglutination*

In this simple direct technique, a cell or insoluble particle is agglutinated directly by antibody. An example is the agglutination of group A red cells by anti-A sera in a simple slide agglutination test. In this way several species of bacteria (grown on culture) can be directly agglutinated and thus definitively diagnosed by specific antibody using a slide agglutination reaction.

Tube agglutination reactions are used to detect specific antibody (in human or animal serum) in the presence of a constant amount of antigen. Classically,the serum to be tested is initially, serially diluted in test tubes, using two fold dilutions such as 1 in 2, 1 in 4 or 1 in 8 etc., alternatively dilutions such as 1 in 10, 1 in 20, 1 in 40 etc., may be used. Essentially, what this means is that 1 part of serum is mixed with 1 part of a diluent in a 1 in 2 dilution, or one part of serum is mixed with 3 parts of diluent in a 1 in 4 dilution and so on. The diluents used are usually normal saline or any other suitable buffer solution. Standardized amounts of known antigen are then added to all the tubes. The tubes are incubated at suitable temperature (usually 37 C), for most tests except for cold reacting antibodies or cold agglutinins which need refrigeration. After a few hours of incubation, agglutination is complete and tubes are examined for clumping. The results are usually expressed as a titer i.e., the highest dilution of the test serum at which a positive agglutination reaction occurs. Important clinical applications of this technique are the **Widal test** for diagnosis of typhoid fever, the **Brucella** agglutination test for Brucellosis and the **Weil Felix** test for Rickettsiosis, to name just three.

(*ii*) *Indirect / passive agglutination*

When a soluble antigen needs to be used in an agglutination reaction, it is often coated onto a carrier particle, thus rendering it particulate. Soluble antigens can be passively adsorbed or chemically coupled to red blood cells of various species, in which case the test is called an indirect (passive) haemagglutination test (IHA or PHA). Many antigens will spontaneously couple with red cells and form stable reagents for antibody detection. *Treponema pallidum* haemagglutination (TPHA) for the diagnosis of syphilis is a widely used test that exploits this principle. Coupling agents vary widely, however, a good example is tannic acid which increases the amount of most protein antigens coupled onto red cells.

Agglutination tests are performed in tubes or microtiter plates which hold serially diluted serum samples. A constant amount of coupled antigen is then added to each serum dilution and

incubated. After a few hours of incubation, a positive reaction is seen as clumping of red cells, a negative reaction is evidenced by settling of red cells at the bottom of the tube or well as a button (Figure 8.12). Passive haemagglutination with protein sensitized cells can detect antibody at concentrations as low as 0.03 μgs/ml.

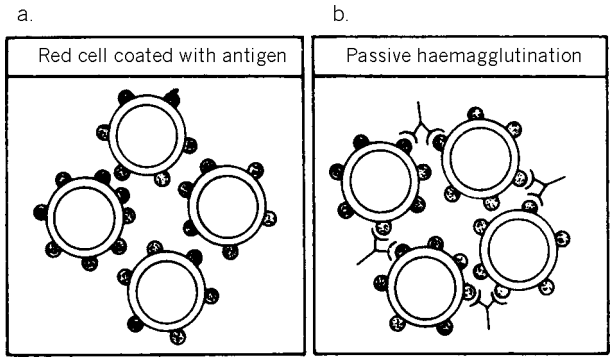


Figure 8.12. Indirect (passive) haemagglutination (a) red cells are coated with antigen (b) specific antibody causes indirect (passive) haemagglutination.

A modification of the above technique that is commonly used involves coupling of antibody onto red cells so as to **detect antigen** in the patient's sample. Since the reactants are reversed the test is sometimes called reversed passive haemagglutination (RPHA).

Passive carriers of antigen, other than red blood cells, have been widely used in serology for the demonstration of agglutinating antibody. Among the commonest ones used are inert particles like latex in the latex agglutination (fixation) test or staphyloccal protien A (Figure 8.14). The latex agglutination test is widely used for detection of Rheumatoid factor. Rheumatoid factor is a 19S IgM antibody (an auto antibody) directed against the patient's own 7S IgG. If latex particles are adsorbed with 7S IgG, when mixed with serum of patient with rheumatoid arthritis, the 19S IgM in these sera react with 7S IgG coated onto latex particles and cause visible clumping of the particles.

A passive haemagglutination test is also used to detect rheumatoid factor. This test is called the **Rose-Waaler test**. Antibodies (IgG) against sheep red cells are raised in rabbits.Human rheumatoid factor (IgM) has been shown to react against the rabbit IgG raised against sheep red cells. This antiserum is also known as amboceptor. Tanned sheep red cells are coated (sensitized) with the amboceptor in sub agglutinating titers. These sensitized sheep cells are allowed to react with serial dilutions of human serum. Rheumatoid factor in human serum will react with rabbit anti sheep IgG coated onto the sheep red cells, to cause clumping of red cells. This occurs by virtue of the fact that rabbit IgG and human 7S IgG cross react (Figure 8.13). Before interpreting the test reading it is important to ascertain that the given patient's serum does not react with normal unsensitized sheep cells, causing non specific clumping.

(*iii*) *Haemagglutination inhibition*

Another category of agglutination involves spontaneous agglutination of red cells by certain viruses. This viral haemagglutination reaction can be specifically inhibited in the presence of antiviral antibody. Thus viral haemagglutination can be used to detect presence of virus; alternatively, the haemagglutination inhibition reaction can be used to detect the presence of

specific antiviral antibody in patient's serum. This happens because viral antigen reacts with antiviral antibody in patient's serum; the haemagglutinating sites on the viral particle thus lie complexed to antibody and are no longer available for reaction with red cells.

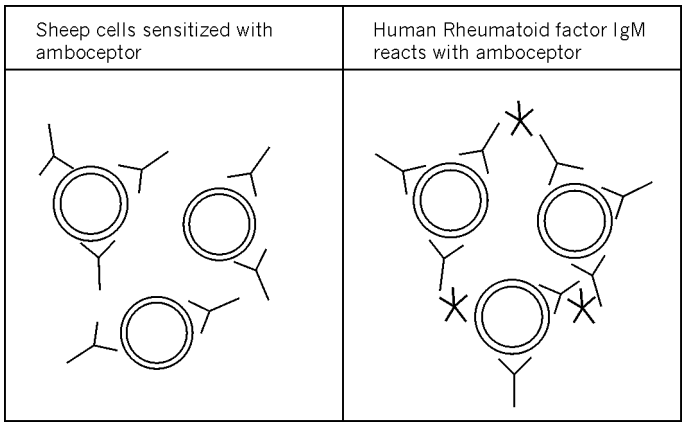


Figure 8.13. Principle of the Rose-Waaler test a) Sheep red cells sensitized with rabbit IgG raised against the sheep red cells (sub agglutinating doses) b) Human IgM (rheumatoid factor) reacts with rabbit IgG causing agglutination of red cells.

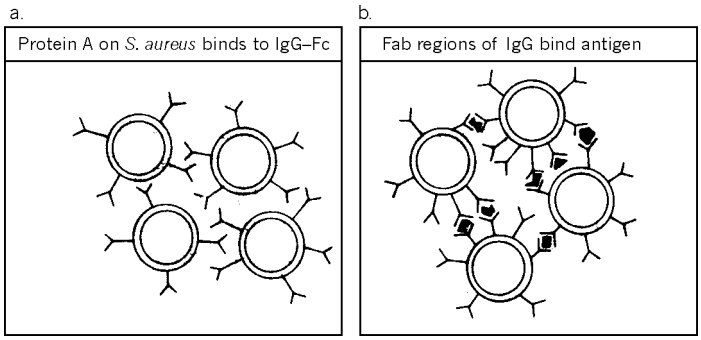


Figure 8.14. Co-agglutination (a) Protein A containing S.aureus binds avidly to the Fc portion of the IgG molecule. (b) The Fab portion of the bound IgG complexes with antigen resulting in clumping of *S.aureus* cells.

Coomb's Test

In some cases a short antibody molecule directed against a deeply located membrane antigenic determinant cannot agglutinate (Figure 8.15 a),as the two Fab arms are not long enough to bridge determinants on apposing red cells. These antibodies are sometimes called "incomplete" or "blocking" antibodies. A good example of such a determinant is the Rh antigenic determinant on red cells. To detect the presence of anti Rh antibodies, already fixed to red cells, an anti human immunoglobulin is added which is directed against the "incomplete" antibody. The addition of this second antibody leads to agglutination (Figure 8.15 b).

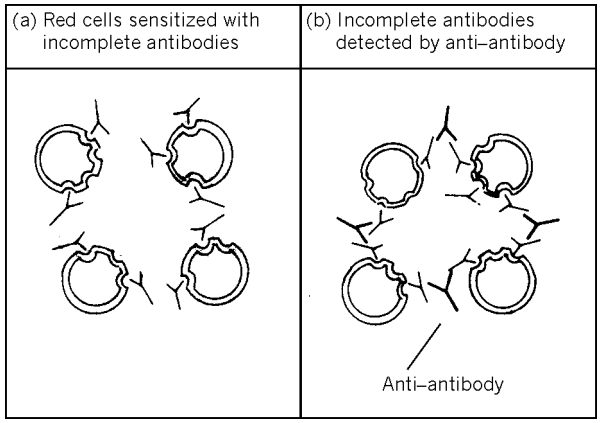


Figure 8.15. The Coomb's test (a) red cells are sensitized with "incomplete antibodies" (b) Anti-antibody reacts with the "incomplete antibody" causing agglutination of red cells.

The Coomb's test is performed in two ways:

(*i*) The **direct Coomb's test** detects immunoglobulin already present on the red cell by addition of the antiglobulin (as described above). The direct Coomb's test is positive in an already sensitized individual, such as the Rh positive infant born to an Rh negative mother. Such an infant's red cells are coated with anti Rh antibody produced by the Rh negative mother against her own baby's Rh positive red cells. The infant is thus at risk of developing haemolytic disease of the newborn.

(*ii*) The **indirect Coomb's test** is a 2 stage reaction for detection of circulating incomplete antibodies against the Rh determinant, which are usually present in the mother's serum after the birth of an Rh positive infant. The patients serum is first incubated with red cells possessing the Rh determinant. The incomplete antibodies react with the Rh determinants and coat the red cells. Once coating has taken place, the antiglobulin is added and the appearance of agglutination indicates the presence of anti Rh antibodies in maternal serum. The Coomb's test is also used in the diagnosis of auto immune haemolytic anaemia.

Complement Fixation Test

The fixation of complement occurs during the interaction of antigen and antibody. Thus the consumption of complement in vitro, can be used as a test to detect and measure antibodies, antigens or both. The test depends on the use of a haemolytic indicator system, consisting of sheep red cells, amboceptor (antibody to sheep red cells) and complement. When added together, sheep red cell antigen complexes with antibody, utilizes complement and resultant red cell lysis occurs (Figure 8.16). The actual test is done in two stages: the first stage consists of adding the test reagents which are antigen and antibody (normally the biologic fluid such as human serum) and a standardized amount complement. If antigen and antibody are specific for each other they will complex and utilise the complement as well. The second stage of the test, is done by adding the sheep cell-amboceptor mixture (also known as sensitised sheep red cells). After a period of incubation the test is read looking specifically for the presence and degree of red cell lysis.

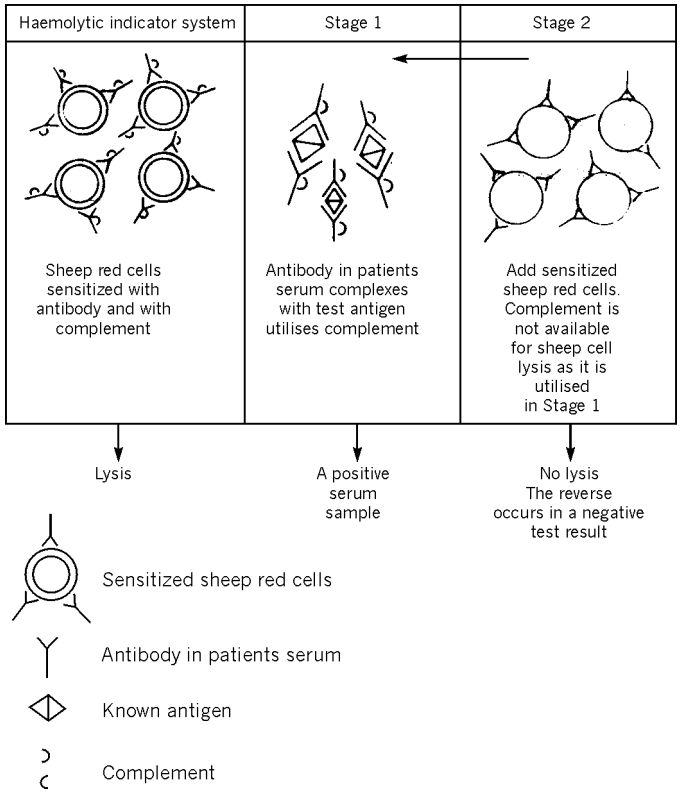


Figure 8.16. The Complement fixation test.

Interpretation

Occurrence of lysis indicates: test antigen and antibody in stage one did not complex, therefore did not utilise complement. Complement was hence freely available for utilisation by the haemolytic indicator system, with resultant red cell lysis. Lysis therefore indicates a negative test reaction since the patient's serum did not contain the antibody in question. The **absence of lysis** indicates: a positive test reaction since antigen was complexed to antibody (present in patient's serum) and utilised most or all of complement. Complement was hence not available to the haemolytic indicator system and sheep red cell lysis did not occur (Figure 8.16). Results are shown as the highest serum dilution showing a positive reaction.

Complement fixation tests have received widespread use in both clinical and research laboratories, though they have now been replaced by less cumbersome immunoassay tests.

Immunoassay Using Labelled Reagents

One of the most important analytic methods developed in recent years is the immunoassay of antigen and antibody using labelled reagents, also known as binder-ligand assays.

The first ligand assay method to be developed was the radio immunoassay (RIA), which was introduced to detect human insulin using anti insulin antibodies. The method was described by Berson & Yalow and in 1977; Yalow was awarded the Nobel prize for her contributions to the field of binder-ligand assays. In the past two decades ligand assays have revolutionized the quantification of hormones, drugs, tumour markers, allergens and a variety of antigens and antibodies associated with infectious agents. The chief goal of an assay is to determine the concentration of some molecule of interest - the analyte, be it an antigen or an antibody.

Radio immunoassay

Radio immunoassays has been used to quantify hormones such as insulin, thyroxine etc, in plasma. This is done by using antibodies raised in rabbits or guinea pigs to the concerned hormones. An initial step in the use of the RIA is to plot a standard graph for each hormone tested. This standardization procedure is described below:

(*i*) For estimation of the hormone X (for e.g.,thyroxine) antiserum to X is raised in rabbit or guinea pig.

(*ii*) Pure hormone X is obtained and radio labelled to yield X*.

(*iii*) The concentration of labelled hormone i.e., X* is titrated so as to react with 70% of binding sites when added to anti X.

(*iv*) A range of known concentrations of unlabelled pure X is added simultaneously with the titrated X* to the antibody and the mixture is incubated.

(*v*) Labelled X* + antibody is separated from free X by the addition of an anti-antibody.

(*vi*) From the amount of bound X* measured out at various X concentrations a curve is constructed. The curve is linear over a relatively limited range, hence the sample containing X needs to be diluted so as to give a reading within this range (Figure 8.17).

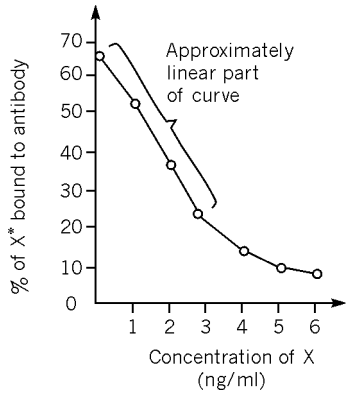


Figure 8.17. Standard curve for hormone assays by RIA.

After the standardization is achieved, the experiment is repeated replacing X in the above step no.(iv) with the patient's sample. The percentage of X bound to antibody is estimated in the presence of the clinical specimen. Using the graph (Figure 8.18), the concentration of the hormone X in the patients sample can be ascertained.

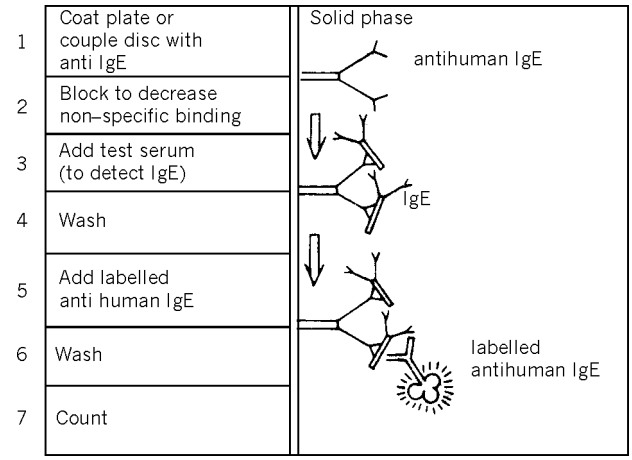


Figure 8.18. The radio-immunosorbent test (RIST) detects total IgE in patients serum.

Solid phase radio immunoassay

Solid phase radio immunoassays may be used to detect both antigen and antibody. A good example to illustrate this is the **radio immunosorbent test (RIST)** which detects total IgE in a patient's serum. The test is usually done in a microtiter poly - carbonate plate with multiple wells. Anti human IgE raised in rabbits in used to coat the well (Figure 8.18), or is coupled via cyanogen bromide to micro-cellulose discs. The solid phase is blocked with non-specific protein to decrease non-specific binding. Dilutions of the patient's serum are added to the coated well. Excess serum is washed off ensuring that human IgE alone, is in the well coupled to anti IgE. The bound IgE is then measured by addition of labelled anti human IgE raised in yet another animal. Measurements of radioactivity in the well are then recorded and quantified using a geiger counter.

The **radio allergo sorbent test (RAST)** measures the amount of IgE to specific antigens (allergens) in the patient's serum. The specific allergen (eg: pollen extract) is coupled to a paper disc in a microtiter well. This is treated with the patients serum, washed and bound human IgE detected with labelled antihuman IgE similar to the procedure used for the RIST (Figure 8.19).

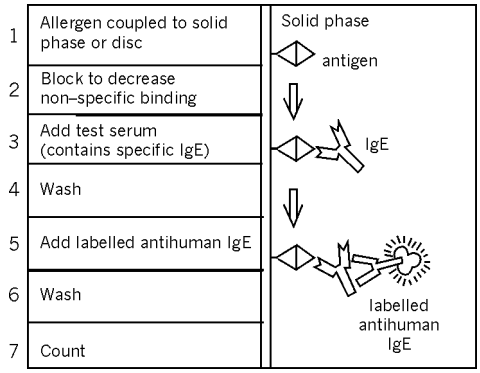


Figure 8.19. The radio allergosorbent (RAST) test to detect specific IgE.

Enzyme labelled immunosorbent assays (ELISA)

The use of radio labelled reagents carries with it the hazards of radioactivity, the problems of safe disposal and the prohibitive cost of reagents and equipment.

Radiolabelled reagents have now been largely replaced by enzyme- labelled antibodies, which when exposed to coloured substrate complexes release the colour. Furthermore, the reaction can be quantified by measuring the density of colour using a simple colourimeter. The basic principle is very similar to the solid phase radi o immunoassay (Figure 8.20 a and b).

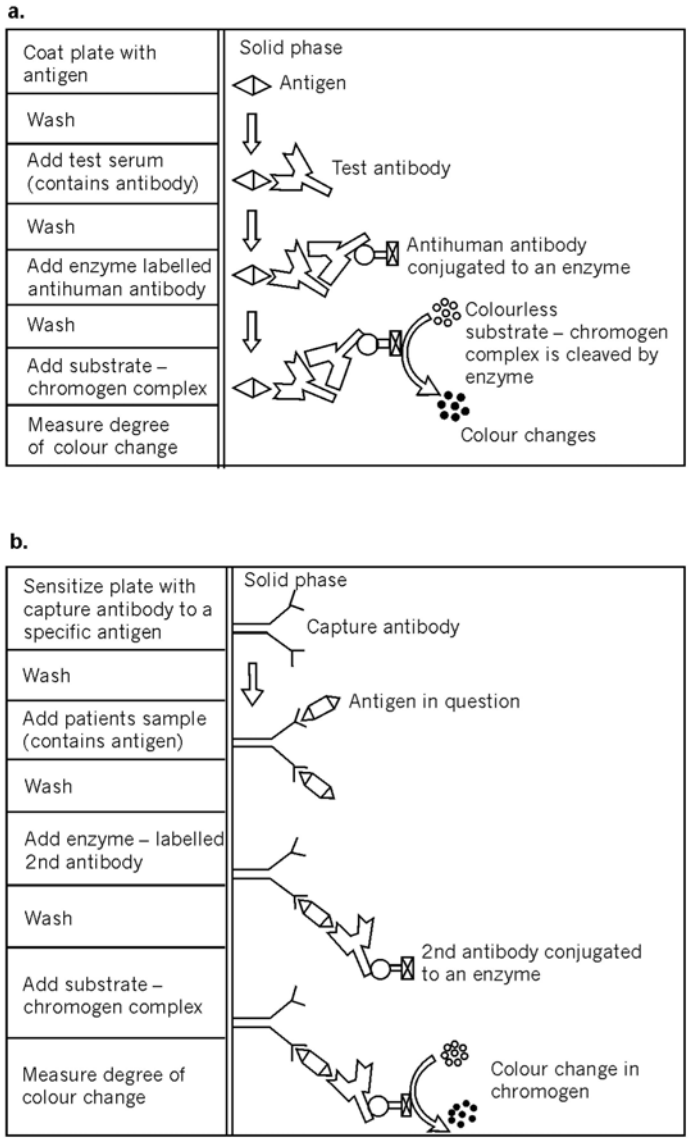


Figure 8.20. (a) The enzyme linked immunosorbent assay (ELISA) to detect antibody (b) The ELISA to detect antigen.

Commonly used enzyme labelled antibodies and their respective colour substrate complexes are given in Table 8.1.

Table 8.1: Enzyme labelled antibodies with respective substrate complexes

Antibodies conjugated to	*Substrates*
Horse radish peroxidase	hydrogen peroxide + ortho phenylene diamine
Alkaline phophatase	p–nitrophenyl phosphate
β– galactosidase	o–nitro–phenyl–β–d– gal actopyranoside

Biotin / avidin enhanced immuno assays

A variation of the ELISA is a system which uses the strong affinity of compounds like biotin and avidin for each other. These assays have been shown to be several hundredfold more sensitive than the RIA, and can therefore be used to detect very small quantities of antigen or antibody. The variation involves biotinylating the second antibody after which avidin conjugated to an enzyme is added, this forms a firm complex which is detected using a substrate-colour compound as in conventional ELISAs.

The ELISA and its many variations are widely used in clinical immunology (Table 8.2). Class specific antibodies (IgG, IgM, IgA or IgE) can also be detected using the appropriate second antibody.

Table 8.2: Applications of the ELISA

For antigen detection
Hepatitis B surface antigen (HBsAg)
HBeAg
For antibody detection
Anti Hbs
Anti Hbc
Antibodies to toxoplasma,
Antibodies rubella and other viruses
Antibodies to HIV 1 and HIV 2

Immunochromatography (Immunocard tests)

Immunoassays have been used in a variety of commercially available immunocard tests. These tests use the principle of immunochromatography. The disposable device consists of chromatographic membrane with a liquid-phase antibody conjugated to a coloured substrate (eg: conjugated mouse monoclonal antibody) to the antigen to be detected. This is called the signal antibody; while the two solid-phase antibodies are a polyclonal antibody to antigen and a polyclonal antibody to mouse immunoglobulin in two stationary antibody zones (Figure:8.21). Patient sample (stool extract for example) is introduced into the device and incubated at room

temperature. The specimen migrates via capillary action along the membrane until it reaches the first stationary specific monoclonal antibody to the antigen in question. The specimen, as it migrates, also dissolves the embedded signal antibody. Antigen binds to the stationary antibody, the signal antibody also binds to antigen. Reaction takes place and a coloured line appears. The excess signal antibody which does not bind to antigen migrates further until it reacts with the polyclonal antibody to mouse immunoglobulin, producing a separate, second coloured line. Thus, two coloured lines on the test stick indicate the presence of antigen. In the absence of antigen in the patient's sample, only one coloured line develops, as a result of the reaction between the signal antibody and the antibody to mouse immunoglobulin. Colour must always be seen at the control line. If no colour is seen, the test has failed and must be repeated. Results are read by comparison of the intensity of colour at the test line with the control line – equivalent colour is positive, darker colour is strongly positive. These tests are now widely available for diagnosis of *Clostridium difficile toxins* A and B, *Helicobacter pylori* faecal antigen tests, pregnancy tests and many other antigen detection tests.

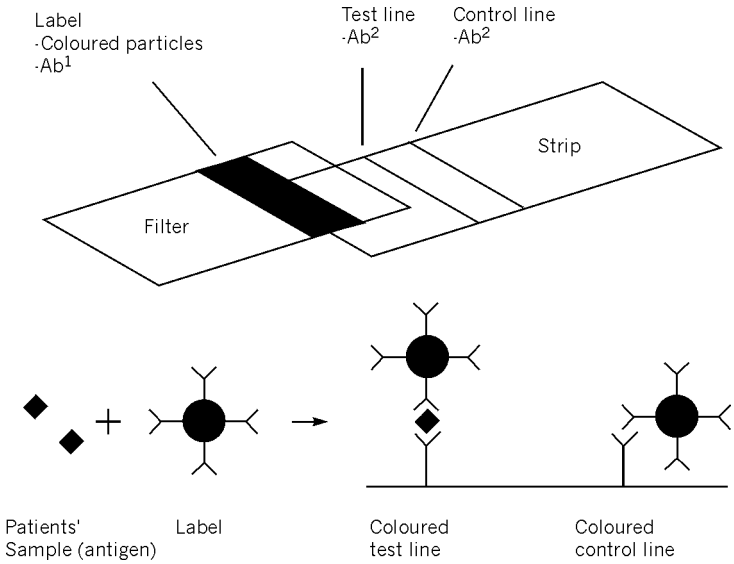


Figure 8.21. Immunochromatography.

DNA immunoassays

DNA enzyme immunoassays have been developed to detect a variety of micro organisms. Commercial kits often use streptavidin coated microwell plates to which is added a biotinylated specific probe based on the gene to be detected (for eg you may want to detect the UreC gene of *H. pylori*). The amplified gene product (by prior PCR) is added to the plate and the duplex DNA detected with an enzyme linked antibody against double stranded DNA. These PCR-ELISAs are becoming very popular diagnostic tools for a variety of micro organisms where it is important to detect the specific pathogenic or antibiotic resistant gene in the organism.

Immuno blotting (Western blots)

Western blots are used to detect antibody targeted to individual antigenic determinants in a crude whole cell antigen mixture. Initially, the antigen mixture is subjected to electrophoretic separation in a gel using, for example, sodium dodecyl sulphate (SDS) poly

acrylamide gel electrophoresis (SDS -PAGE). The separated protein bands are then transferred or blotted onto nitro-cellulose sheets by transverse electrophoresis, where they bind nonspecifically. These nitro-cellulose sheets are then incubated with the patient's serum, when antibodies are allowed to bind to individual protein antigens. Bound human antibody is then detected using enzyme labelled or radio labelled antihuman immunoglobulin, much like the system used in an ELISA or RIA (Figure 8.22).

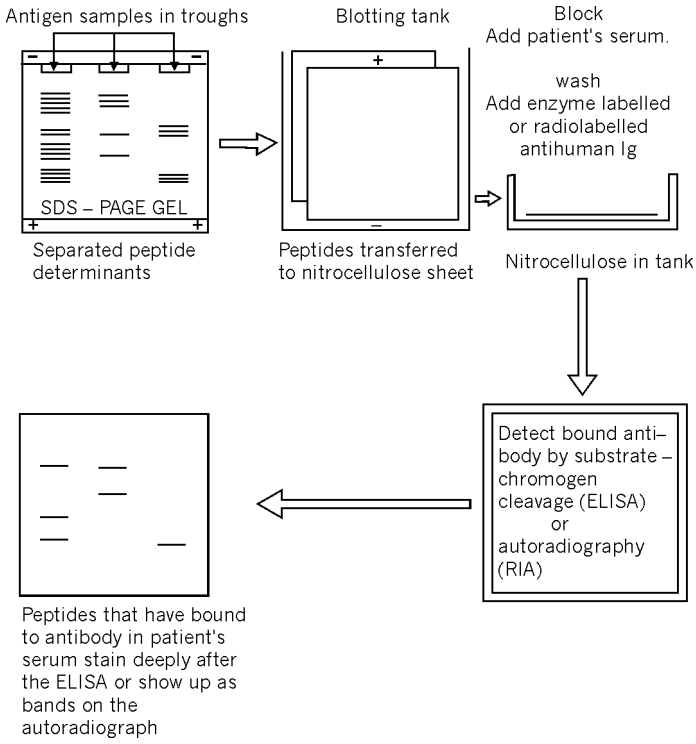


Figure 8.22. Immunoblotting (Western blots).

The recombinant immunoblot assay (RIBA) used for the diagnosis of Hepatitis C uses the Western blot principle; serum is incubated on nitrocellulose strips on which four recombinant viral proteins are blotted. Colour changes indicate that antibodies have adhered to the proteins. An immunoblot is considered positive if two or more proteins react.

Immunohistochemical techniques

Immunofluorescence is essentially a histochemical or cytochemical technique for detection and localization of antigens. Fluorescent dyes such as fluorescein and rhodamine can be coupled to antibodies without destroying their specificity. Such conjugates can combine with antigen present in a tissue section and the bound antibody can be visualized by means of a fluorescence microscope. In this way the distribution of antigen throughout a tissue and within cells can be demonstrated.

Immunofluorescence tests can be either direct or indirect (Figure 8.23 a and b). In the **direct immunofluorescent** test the antibody to the tissue substrate is itself conjugated with the fluorescent dye and applied to cells or tissues fixed on a slide. Alternatively, antigens can be spotted onto a slide and detected by direct fluorescent labelled antibody (Figure 8.23a).

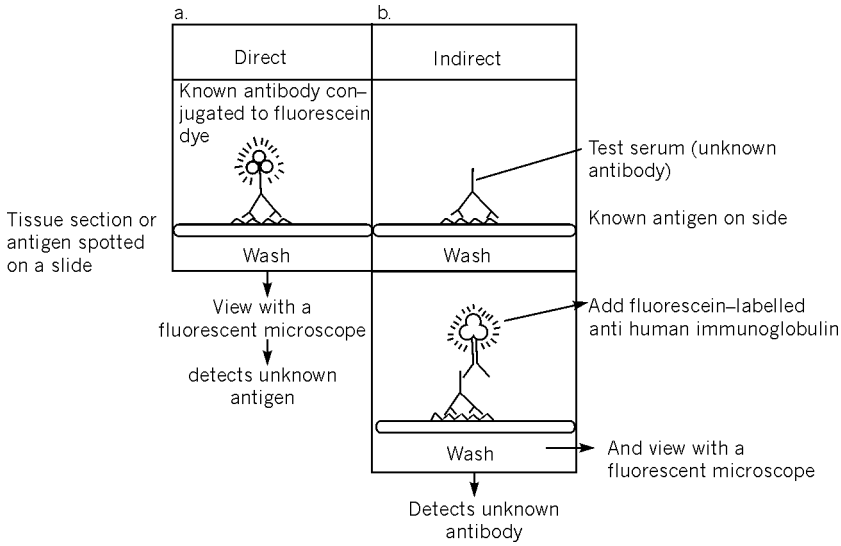


Figure 8.23. The basis of immunofluorescence (a) direct immunofluorescence (b) indirect immunofluorescence.

The i**ndirect immunofluorescence** test is a double layered technique (Figure 8.22 b). The unlabelled antibody is applied to the cell preparation,tissue substrate or antigen spotted onto a slide. This antibody is then treated with an anti human immunoglobulin labelled to a fluorescent dye. When visualized under a fluorescent microscope, bound, labelled anti globulin fluoresces, emitting a characteristic colour.

Immunofluorescence techniques can be used to detect both antigen and antibody in clinical material. Clinical applications of immunofluorescence are given in Table 8.3.

Table 8.3: Clinical applications of Immunofluorescence

Identification of T and B cells in blood.
Detection of immunoglobulins in tissues.
Detection of complement components in tissues.
Detection of specific tissue fixed antibody.
Rapid identification of micro organisms in tissue or culture e.g., *Chlamydia trachomatis*
Identification of tumour specific antigens.
Identification of transplantation antigens.

Cytokine Immunoassays

Detecting an immune response to the pathogen has been a successful method of diagnosis of infection for many years. However, there are a number of limitations to using antibody detection for the diagnosis of acute infection: it is difficult to differentiate past infection from acute current infection; and sometimes the antibody response is insufficient and cannot be used as a diagnostic method. A classical example is tuberculosis(TB) where antibody detection has not played a role in diagnosis.

Our understanding of the immune response mechanisms tells us that antibodies are invariably formed with T cell help. So where there are antibodies there must also be activated T cells. T cells, in particular, control the fight against intra-cellular pathogens such as *M. tuberculosis*, viruses and tumour antigens. Until recently, T cells have been difficult to detect. The T-spot® or ELISPOT® technology is a simple method that can be used in routine laboratories to detect pathogen specific T cells in blood.

When white cells from the patients blood are exposed to a specific antigen (for e.g, *M. tuberculosis* antigen), the T lymphocytes produce γ interferon as a result of activation by antigen. This γ interferon is captured by antibody to γ interferon which is pre-coated in wells of a micro-titre plate. By a modification of the ELISA method the captured γ interferon is detected by a second anti-γ interferon antibody conjugated to a suitable substrate-indicator complex (Figure: 8.24). Cytokine immunoassays are being developed for a number of other viral and tumour antigens. The ELISPOT method for tuberculosis is sensitive and specific, is able to detect active and latent TB in immunocompetent as well as immunosuppressed individuals and does not cross react with BCG.

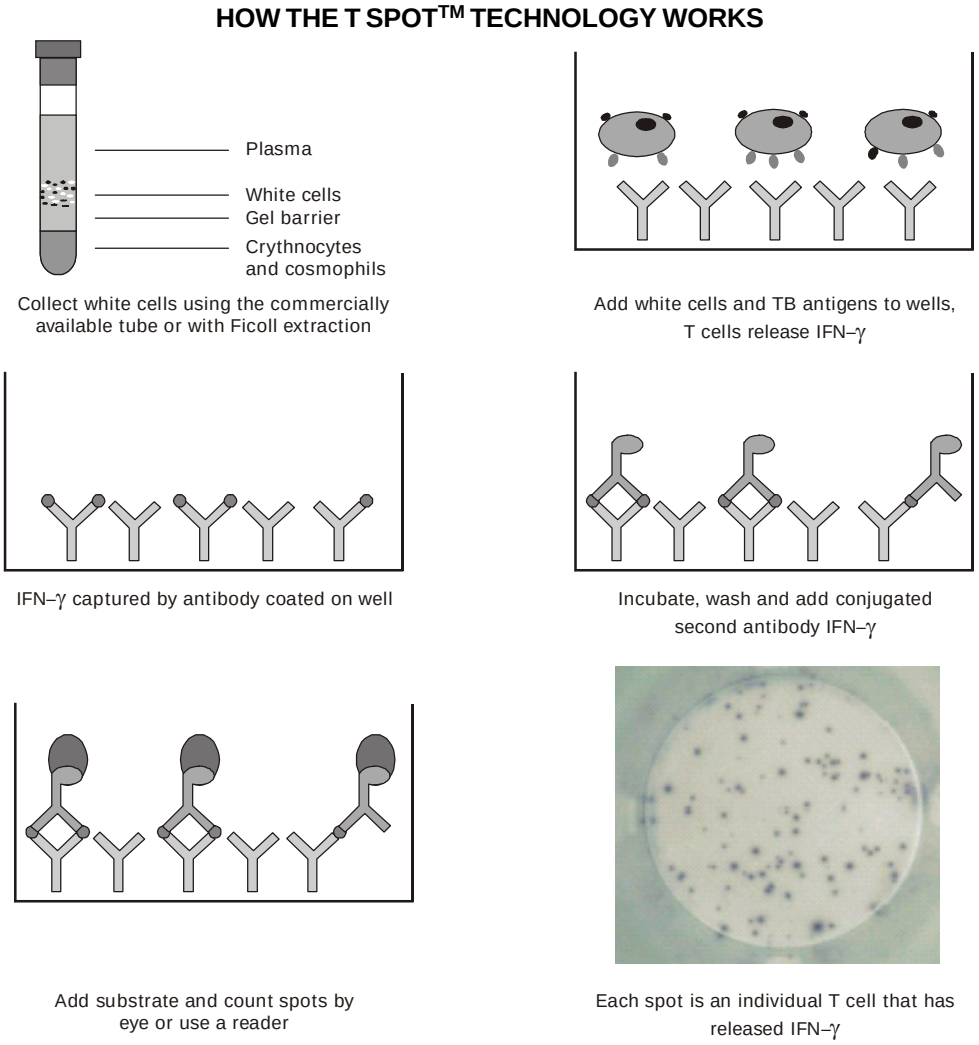


Figure 8.24. Cytokine immunoassay– ELISPOT® techonology

Predictive Theory and Immunologic Testing

When any test is used to make a decision there is some probability of drawing an erroneous conclusion. This divides results into four categories:

- true positives (a+c)
- true negatives (b+d)
- false positives (b)
- false negatives (c)

		Gold Standard test	
		Positive	Negative
New test	Positive	a	b
	Negative	c	d

Diagnostic sensitivity of a new test is the proportion of true positives (as defined by a gold standard test) correctly identified by the new test. Sensitivity = a / a+c.

Diagnostic specificity is the proportion of true negatives (as defined by a gold standard test) correctly identified by the new test. Specificity = d / b+d.

The positive predictive value (PPV) of the new test is the proportion of individuals showing a positive result with the new test who actually have the disease. PPV = a / a+b.

The negative predictive (NPV) of the new test is the proportion of individuals showing a negative result with the new test who do not actually have the disease. NPV = d / c+d.

CHAPTER – 9

MONOCLONAL ANTIBODIES

For many years antibodies have had a major role in the diagnosis of a wide variety of diseases. Immunologic assays are used extensively, as discussed in the previous chapter, in diagnostic laboratories to detect and quantify drugs, bacterial and viral products, tumour antigens and circulating immunoglobulins. These assays have been complicated because of antibodies of restricted reactivity and the heterogeneity of all antisera obtained by conventional methods. These methods, most commonly involve injecting whole antigen, with or without an adjuvant into animals such as rabbits, mice, goat, sheep etc. Several B cell clones are involved, each producing a slightly different antibody molecule to the myriad antigenic determinants, encompassing the whole antigen. This phenomenon is referred to as heterogeneity of the antiserum and occurs even when apparently pure antigen and inbred strains of animals are used. The affinity and quantity of the antibody varies even from one bleed to another. Such an antiserum, secreted by many antibody producing B cell clones, is termed a polyclonal antiserum. The variability of sub specificities and cross reactivities in polyclonal antisera have plagued immunologists all along and forced them to remove unnecessary antibodies by repeated absorption, thereby depleting the original antibody content and strength of the serum. In other cases, certain antisera are rare to come by and reference laboratories face the arduous task of keeping rare reference sera in constant supply.

The Genesis of Monoclonal Antibodies

As it often happens in science, the solution grew out of a series of basic and completely unrelated experiments. To examine the genetic control of immunoglobulin production, a number of laboratories had been attempting to fuse mouse myeloma cells to each other or other cell lines. In the course of such studies Georges Kohler and Cesar Milstein from Cambridge, England showed that cultured mouse myeloma cells could be fused to normal spleen cells of animals immunized with an antigen, in their case, sheep red cells. Since these workers hybridized single cell mixtures, they concluded that subsequent antibody forming cell lines were clones of (progeny of) this one cell (mouse myeloma) to one cell (normal spleen cell) hybridization. These clones were, in fact, producing antibody to a single determinant by a single hybridized B cell, hence the term monoclonal antibody. These cell lines grew continuously in culture because of the cancerous myeloma hybrid, forming little tumour like masses in vitro - giving rise to the term hybridoma. These hybridomas continuously secreted antibody; they could be frozen away, recovered and injected into the peritoneal cavity of mice, where they grew and produced an ascites which yielded large amounts of antibody. The yields are to the tune of upto a 100 ugs of antibody/ml of culture, and 10 mgs/ml in serum or ascitic fluid of tumour bearing mice . These findings have exceeded the wildest dreams of immunologists and have revolutionized serology. For this invaluable contribution to the progress of medicine, Kohler and Milstein shared the Nobel Prize in 1984, with Niels Jerne who introduced the theoretical concept of clonality of the immune response.

The Principle of Monoclonal Antibody Production

Mice are immunized with the whole antigen of interest (containing, let us say, two epitopes: a and b), and given another injection to obtain a secondary response (Figure 9.1). Two to four days later the spleen is removed and teased apart to form a suspension of spleen cells. Some of these spleen cells will be committed to produce antibodies to epitope "a", others to epitope "b". The spleen cells are mixed with mouse myeloma cells that have been previously adapted to grow in continuous culture. Polyethylene glycol (PEG) is added to promote the fusion between cell membranes and the cells are suspended in tissue culture medium. The mouse myeloma cells used are variants, in that, they lack the enzyme hypoxanthine phosphoribosyl transferase ($HPRT^-$) and are also non-secretors of immunoglobulin (Ig^-). The spleen cells on the other hand, being normal, possess both the enzyme ($HPRT^+$) and the ability to secrete immunoglobulin (Ig^+). Only one in every 2×10^5 spleen cells actually forms a viable hybridoma; it is therefore necessary to eliminate the unfused cells to allow recovery of the hybrid. This is done by growth in selective medium containing: H – hypoxanthine; A – aminopterin; T – thymidine.

Because myeloma cells lack the enzyme HPRT, they cannot use exogenous hypoxanthine to synthesize purines. Aminopterin blocks endogenous synthesis of purines and pyramidines, hence myeloma cells die after a period of time. Spleen cells being normal, diploid cells do not have a very long life span in culture and will also die before long. However, a hybrid between a myeloma cell and a spleen cell which possesses the enzyme HPRT, is able to synthesize purines and pyramidines,and survives. The hybrids are thus immortalized because the myeloma cell line contributes the property of growth in continuous culture and the spleen cell provides the hybrid with HPRT. Non immunoglobulin producing myeloma cells are essential as the immunoglobulin desired is the one that the spleen cell is committed to produce - myeloma immunoglobulin or hybrid immunoglobulins would pose many obvious problems.

The mixture of spleen and myeloma cells grows in wells of a microtiter dish. Two to four weeks after fusion, hybridomas become visible on gross examination and the supernatant of these clones are examined for specific antibody to various individual antigenic determinants usually by RIA or ELISA; since these are sensitive, quick and reliable assays. Clones producing antibody are grown in mass culture and recloned. These can either be frozen away or injected to induce tumours in mice. The resultant ascitic fluid formed in the mice yields very high concentrations of antibody.

Advantages of Monoclonal Antibodies

Once a hybridoma has been established, the exact same antibody can be produced by different groups of workers indefinitely. This eliminates differences in results between laboratories. This is important in the case of diagnostic reagents. Besides, large amounts of antibody can be produced in this way, providing a steady supply of reagents for diagnostic immunoassays, blood grouping and HLA typing.

Drawbacks of Monoclonal Antibodies

Since monoclonal antibodies produce antibodies to a single determinant, they do not form the lattice necessary for precipitation and so cannot be used in precipitation assays, radial immunodiffusion, immunoelectrophoresis or agar gel diffusion. Some antibodies generated may not fix complement and hence are not useful in complement fixation tests.

Very high titer antibodies also pose certain difficulties. They may recognize antigens of a low reactivity as in recipients of multiple transfusions or multiparous women. Interpretation of these results becomes confusing and difficult. This kind of cross reactivity cannot be removed by absorption as in the case of conventional antisera.

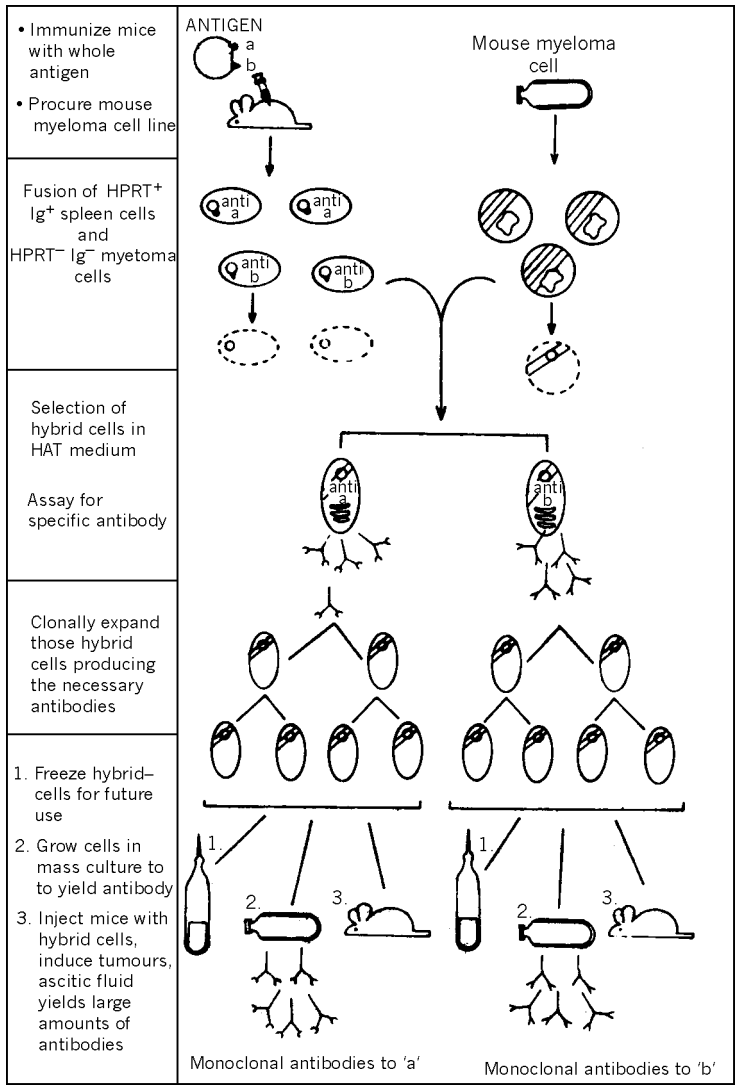


Figure 9.1. The principle of monoclonal antibody production.

The Production of Hybrid Antibody Molecules

With current technology it is possible to introduce DNA into cells and have it expressed as if it were part of the cells own genetic apparatus. By this process called DNA transfection, the immunoglobulin gene DNA can be introduced into myeloma cells. In this manner not only can

myeloma cells produce monoclonal antibodies, the DNA can be custom - altered to yield modified monoclonal antibodies. For example, recombinant antibodies can be produced with the desired antigen binding capacity and fused to a portion of a molecule with enzymatic function. Such a molecule could be used in immunoassays with no need for second antibody techniques. The gene for a specific antigen binding region (say, a tumour antigen) could be coupled to the gene for a toxin, so that the final molecule could be used as a specific toxin to kill tumour cells. Using similar methodology, investigators have generated monoclonal antibodies that are hybrids of mouse and human immunoglobulin molecules. In these molecules, the antigen combining portion is from the mouse gene and the C-region is from the human gene. This methodology may be useful in cases where it is necessary to have human immunoglobulin molecules, and where it is not possible to either immunize humans or to generate an in vitro antibody response with human cells.

Some Applications of Monoclonal Antibodies

Besides a source of pure antibody for diagnosis of infectious diseases, monoclonal antibodies provide a means of:

(*i*) *Enumeration of human lymphocyte sub populations*

Anti-CD3 identifies all mature T cells, anti-CD4 identifies subsets containing T helper cells and anti-CD8 identifies cytotoxic T cells.

(*ii*) *Analysis of viral antigens*

Monoclonal antibodies are used in the classification and diagnosis of viral diseases. The best example of this is the dissection of the antigenic structure of the influenza virus. Monoclonal antibodies have been used against the haemagglutinin glycoprotein that is exposed on the surface of the virus and these antibodies have also been found to neutralize the virus. In this way, every antigenic drift or shift can be identified. Monoclonal antibodies are used to map areas of antigenic variation on the virus particle. Such functional mapping has also been done for polio virus and certain reoviruses.

(*iii*) *Analysis of putative protective antigens*

Monoclonal antibodies can define antigen structure and help identify certain antigenic determinants that evoke a protective immune response. Non immunodominant determinants can also be identified and their reactivity analysed. With the help of monoclonal antibodies synthetic peptides have been produced as subunit vaccines, with configurations corresponding to the protective antigen.

(*iv*) *Analysis of immunologically competent cell surface molecules*

Monoclonal antibodies can be used to map and study the various regions of an immunoglobulin molecule and the basis for immunoglobulin variability. Anti CD8 inhibits killing by cytotoxic T cells. The Anti Ia molecule inhibits T cell responses to macrophage processed antigen. A cocktail of anti CD3 + complement kills T cells in human bone marrow and can be used to prevent graft versus host reaction.

(*v*) *Blood grouping*

Anti blood group monoclonals provide a more reliable standard reagent than conventional antisera.

(*vi*) *HLA typing*

Monoclonal antibodies provide a reliable means of HLA antigen detection using individual specificities to the A,B,C and DR loci.

(*vii*) *Diagnosis of Cancer*

Antibodies have been generated that distinguish between malignant and normal cells. Malignant melanoma cells and acute lymphocytic leukemia cells are among the few that can be differentiated. Radioactive anti carcino-embryonic antigen is used to localize colonic tumours or secondaries on scanning. It may be possible to deliver cytotoxic drugs conjugated to monoclonal antibodies right into tumour specific cell types - the new "magic bullet" therapy.

(*viii*) *In autoimmunity and immune deficiency*

Imbalances in the ratio of T helper and T suppressor subsets indicate immunodeficiency or autoimmunity. These states are accurately monitored by monoclonals against specific T cell antigens. Monoclonal antibodies to the TSH* and ACH* receptors are used to study Graves disease and myasthenia gravis.

(*ix*) *In the control of fertility*

Using monoclonal antibodies against hormones and reproductive tract antigens such as HCG*, LH* and LHRH*, attempts are being made to control fertility.

(*x*) *Monoclonal mutants*

Mutants lacking Fc structures are used for defining biologic roles of Fc domains and for in vivo neutralization of toxic drugs, for example in cases of digoxin overdose.

Hybridoma technology has not only improved the quality and discriminating power of diagnostic and investigative serology, it promises to provide new reagents that will be useful in the diagnosis and treatment of many disease processes.

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*TSH : Thyroid stimulating hormone

ACH: Adrenocortico hormone

HCG: Human chorionic gonadotrophin

LH: Luteinising hormone

LHRH: Luteinising hormone releasing hormone

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CHAPTER – 10

THE MAJOR HISTOCOMPATIBILITY COMPLEX

The discovery of the major histocompatibility complex in humans arose from studies of transplantation of tumours and grafts in mice. It was found that inbred mice of strain A would reject skin grafts from inbred mice of strain B and vice versa, by producing antibodies against membrane antigens on the foreign grafted tissue. These membrane antigens are encoded by genes on chromosome 17 of the mouse, in a region designated H2.

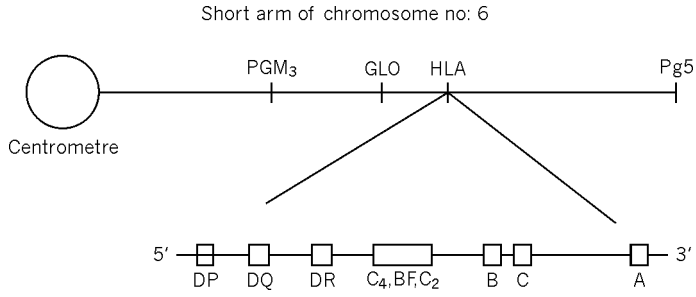
In humans evidence for the existence of similar loci(a locus is a position on the chromosome where a given gene may be found) dates from the mid 1950s when leuko agglutinating antibodies (antibodies that agglutinate leukocytes), were found in recipients of multiple transfusions and in the sera of multiparous women. Such sera were found to agglutinate or lyse leukocytes from some persons but not others. The chromosomal region analogous to the mouse H2 constitutes the "major histocompatibility complex" (MHC), in all species found to possess it. In humans, this region and the antigens encoded by this region are both known by the acronym HLA (human leukocyte antigen). Consequently, the terms MHC and HLA are often used interchangeably.

The vital role played by HLA antigens in tissue and organ transplants was soon appreciated. However, in 1973 certain HLA antigens were found to be associated with specific diseases in high proportion. In addition, it was realized that the major histocompatibility complex regulates several aspects of the human immune response.

Nomenclature and Genetic Organization of the MHC

The nomenclature of the MHC/HLA system is devised by the HLA Nomenclature Committee under the auspices of the World Health Organization. An International Workshop meets every year to update the description and nomenclature of the MHC.

The entire histocompatibility complex occupies a segment on the short arm of chromosome 6. Though the terms HLA and MHC are sometimes used interchangeably, the acronym HLA is more often used to precede the individual antigens within the complex for example, HLA – A, HLA–B, HLA – C, etc. Figure 10.1 schematically depicts the current concept of the MHC, showing the genetic regions containing the HLA loci in relation to each other. The officially recognized genetic loci are HLA–A, HLA-B, HLA–C, HLA – E, HLA – F, HLA–G, H, J, K, L; together with the HLA–D region. The HLA-D region consists of the following loci : HLA–DR (D-related) HLA –DQ and HLA–DP. More HLA related loci are continually being and identified. Several additional genetic regions have been linked to the HLA complex. The complement region, which has been mapped between the HLA–B and HLA–DR regions contains genes determining the complement components C2 and C4 of the classical complement pathway and properdin and factor B F of the alternative pathway.



Figuere 10.1. Genetic organization of the HLA system

In the upper part of the figure the position of the HLA complex, on the short arm of chromosome no: 6, is shown in relation to the other markers: PGM_3= phosphoglucomutase 3; GLO = glyoxylase; Pg5 = urinary pepsinogen. The lower part of the figure shows the expanded version of the HLA complex. Class I loci are the HLA–A, HLA–B and HLA–C regions. A cluster of closely linked complement genes: C4, BF and C2 lies next to the HLA–B locus. The three class II loci are termed HLA–DP, HLA–DQ and HLA–DR from the 5' to the 3' end.

At each locus, one of several alternative forms (alleles) of a gene may be found. Officially recognized alleles at each locus are designated by the locus and a number, for example, HLA–A1, HLA–A2 etc., are the allelic (alternative forms) of the HLA–A locus that may exist. Alleles that have been tentatively assigned to a given locus, but are not yet officially recognized are designated by a w (for workshop) placed before the number for example, HLA–DR w1. The HLA system is extremely polymorphic, having multiple different alleles at each known locus; for instance there are 30 or more different alleles at the HLA–A locus and at least 60 at the HLA–B locus. Each allelic gene determines a different gene product or cell surface antigen which is also given the same number. In several instances HLA antigens initially thought to be a single antigen, have been found to be a group of 2 or 3 closely related antigens. They are identified numerically and in brackets for eg., HLA– A25(10), 10 being closely related to 25.

The combination of alleles at each locus on a single chromosome is usually inherited as a unit. This unit is referred to as the haplotype. Since we inherit one chromosome from each parent we have two HLA haplotypes. Because all HLA genes are co-dominant, both alleles, one each from the maternal and paternal chromosomes, at a given HLA locus are expressed, and 2 complete sets of HLA antigens can be detected on cells. Hence an individual can be typed: HLA–A1, A11 and HLA–B27, B13 and so on.

Linkage Disequilibrium

This is a phenomenon that occurs when certain combinations of alleles are found with a frequency far exceeding that expected from pure random mating. As an example HLA–B8 and HLA –A1 are found together six to 21 times more often in the same individual in some population groups, than would be possible by pure co incidental association. The clinical significance of linkage disequilibrium is as yet unclear. However, the mechanism responsible for linkage disequilibrium remains the subject of many speculations. Currently, the most preferred explanation postulates that certain combinations of alleles at different HLA loci are advantageous to the survival of an individual. The precise nature of this advantage has not been defined, but

it could represent an enhanced resistance to certain infections or diseases, especially those that are prevalent in the environment of a given population.

Antigens of the Major Histocompatibility Complex (MHC)

Based on their tissue distribution and structure, the MHC antigens have been divided into two classes. The **Class I MHC antigens**, also termed the classic histocompatibility antigens include the HLA – A; HLA – B; HLA – C series.

The Class II MHC antigens, analogous to the Ia antigens in the mouse include the HLA – DR; HLA – DQ; HLA– DP antigens.

HLA typing

Lymphocytotoxicity

Availability of monoclonal antibodies to a variety of HLA antigens have made serological testing a popular method of HLA typing. Using the lymphocytotoxicity assay, lymphocytes are added to anti-sera which may or may not have antibodies directed to HLA antigens (shown schematically in Figure 10.2).

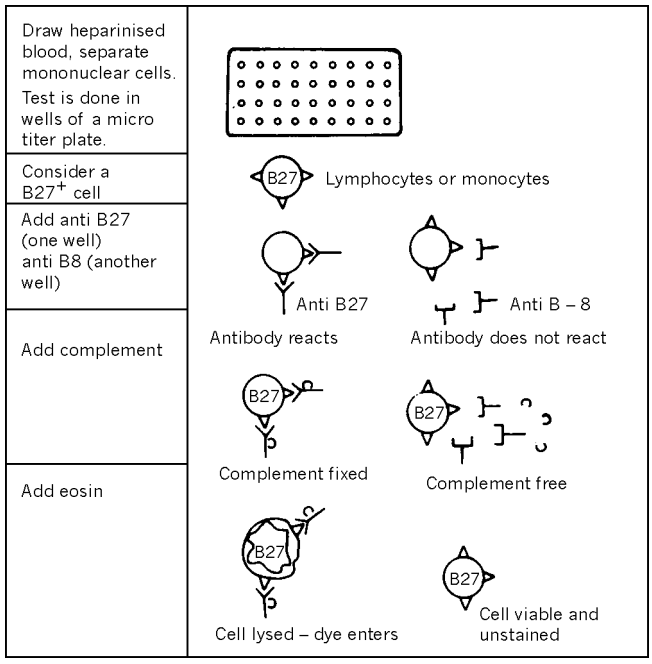


Figure 10.2. Typing for HLA Class I antigens: Microcytotoxicity assay. The above reaction patterns allow the cell to be typed as HLA B27 +; HLA $B8^{-}$.

If the serum contains an antibody specific to an HLA (Class I or Class II) antigen on the lymphocytes, the antibody will bind to this HLA antigen. Complement is then added. The complement binds only to positive cells (i.e., where the antibody has bound) and in doing so,

causes membrane damage. The damaged cells are not completely lysed but suffer sufficient membrane damage to allow uptake of vital stains such as eosin or fluorescent stains such as ethidium bromide. Microscopic identification of the stained cells, indicates the presence of a specific HLA type.

The cells used for the test are lymphocytes because of their excellent expression of HLA and ease of isolation compared to most other tissues. The most important use of this test is to detect specific donor-reactive antibodies present in a potential recipient prior to transplantation.

Historically, this test, using antisera of known specificity has long been used to type for HLA Class I and Class II antigens,. However, the problems of non-availability of certain antibodies has led to the introduction of DNA based methods. Currently, many laboratories have changed to molecular genetic methods for HLA Class typing.

Mixed Lymphocyte Reaction (MLR)

The HLA - DR and HLA - DQ antigens are typed using a serologic procedure similar to the assay for Class I antigens. Instead of using the patients mononuclear cells, these assays are done on purified populations of B lymphocytes. The typing sera are pretested to make certain that they do not detect the Class I antigens. The HLA - DR antigens also elicit a reaction called the **mixed lymphocyte reaction (MLR)**. This occurs when lymphocytes from one individual are cultured with those from another. If these two lymphocyte populations possess differing (heterozygous) HLA - DR antigens, the cells are stimulated to divide. They become metabolically very active in the presence of a foreign HLA - DR antigen and begin to synthesize excessive amounts of DNA. This phenomenon is called **blast transformation**. During blast transformation there is increased uptake of thymidine into the cells to facilitate increased DNA synthesis. If the thymidine can be labelled, as in 3H thymidine, the blast transformation can be measured by following the rate of uptake of 3H thymidine. In an MLR, a panel of known HLA - DR cells called the **homozygous typing cells (HTC)** is used. These stimulator cells are irradiated or treated with mitomycin C to prevent their proliferation in response to the unknown cells. The responder cells of the individual to be typed are cultured with the HTC (Figure 10.3). If the responder cells, after incubation with the stimulator cells, display excessive DNA synthesis, then it is concluded that the patient's cells are not of the same type as the HTC. If only base line DNA synthesis occurs, than the patients cells and the HTC are homozygous (similar).

An important use of the MLC is in its use as a "cellular crossmatch" prior to transplantation especially for bone marrow transplants. By testing the prospective donor and recipient, an invitro transplant model is established which is an extremely useful indicator of possible rejection or Graft versus Host reaction.

Molecular typing techniques

RFLP

Restriction Fragment Length Polymorphism (RFLP) methods rely on the ability of certain enzymes to recognise exact DNA nucleotide sequences and to cut the DNA at each of these points. Thus the frequency of a particular sequence will determine the lengths of DNA produced by cutting with a particular enzyme.

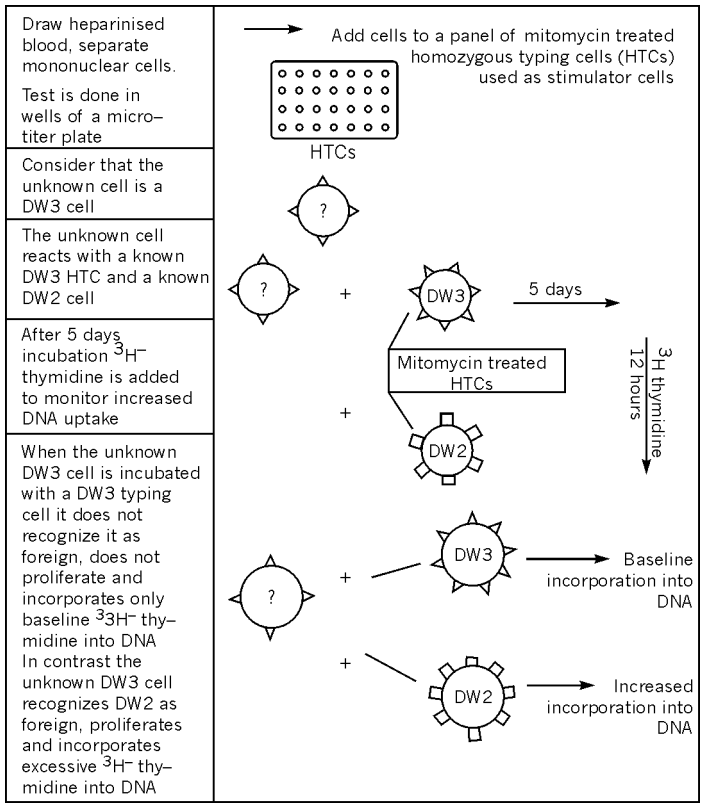


Figure 10.3. Typing for HLA Class II antigens: Mixed lymphocyte reaction. The above reactions allow the cell to be typed as HLA $Dw3^{+}$; HLA-$Dw2^{-}$.

The DNA for one HLA (Class II) antigen, e.g., DR15, will have these particular enzyme cutting sites (or "restriction sites") at different positions to another antigen, e.g., DR17. So the lengths of DNA seen when DR15 is cut by a particular enzyme, are characteristic of DR15 and different to the sizes of the fragments seen when DR17 is cut by the same enzyme.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a recently developed and revolutionary new system for amplifying the DNA nucleotide sequence of a particular region of interest in any individual. Very small amounts of DNA can be used as a starting point. Sequencing DNA is now available using computerised and automated methodology.

The first step in this technique is to obtain DNA from cells of an individual. The double stranded DNA is then denatured by heat into single stranded DNA. Oligonucleotide primer sequences are then chosen to flank a region of interest. The oligo- nucleotide primer is a short segment of complementary DNA which will associate with the single stranded DNA to act as a starting point for reconstruction of double stranded DNA at that site.

If the oligonucleotide is chosen to be close to a region of special interest like a hypervariable region of HLA-DR then the part of the DNA, will be amplified when DNA polymerase and deoxy-ribonucleotide triphosphates are added. From one copy of DNA it is thus possible to make two. Those two copies can then, in turn, be denatured, reassociate with primers and produce four copies. This cycle can then be repeated until there is sufficient of the selected portion of DNA to isolate on a gel and then sequence or type.

There are a number of PCR based methods in use. For example:

Sequence Specific Priming (SSP)

In this test, the oligonucleotide primers used to start the PCR have sequences complimentary to known sequences which are characteristic to certain HLA specificities. The primers which are specific to HLA-DR15, for example, will not be able to instigate the PCR for HLA-DR17. Typing is done by using a set of different PCRs, each with primers specific for different HLA antigens.

Sequence Specific Oligonucleotide (SSO) Typing

By this method, the DNA for a whole region (e.g., the HLA DR gene region) is amplified in the PCR. The amplified DNA is then tested by adding labelled (e.g., Radioactive) oligonucleotide probes, which are complementary for DNA sequences, characteristic for certain HLA antigens. These probes will then "type" for the presence of specific DNA sequences of HLA genes.

In general, because we possess 2 haplotypes each and because HLA expression is co-dominant it possible to type 2 antigens from each locus. Occasionally only one antigen can be typed when it is reported as HLA-B27; B- for example. Such a report could mean that the other HLA-B antigen was homozygous with the typed antigen or that typing was not possible by the methods available.

Uses of HLA Typing

HLA typing is used primarily for determination of HLA compatibility prior to transplantation, for paternity testing, for anthropologic studies and to establish HLA-disease associations.

Structure and function of the MHC antigens

MHC Class I Antigens

The MHC Class I antigens consist of two polypeptide chains held together non-covalently (Figure 10.4). One chain (the alpha chain), is heavy and glycosylated (44,000 daltons).The extra cellular portion of this Class I heavy chain is divided into three domains, designated $\alpha 1$, $\alpha 2$ and $\alpha 3$ looped together by disulphide bonds (Figure 10.4). The extracellular portion has an N terminal and is hydrophilic, the transmembrane portion is hydrophobic, the intra cellular portion is hydrophilic and has the carboxy terminal. The other chain is a small protein (11,500 daltons) known as $\beta 2$–microglobulin which is encoded by a gene on chromosome 15. Both the $\beta 2$ microglobulin and the $\alpha 3$ domain of the heavy chain of the MHC Class I antigen are relatively non- polymorphic and demonstrate considerable amino acid homology with the CH3 portion of the constant region of the IgG molecule. X-ray diffraction studies of the HLA-A2 molecule have shown that the heavy chain domains α 1 and α 2 are most

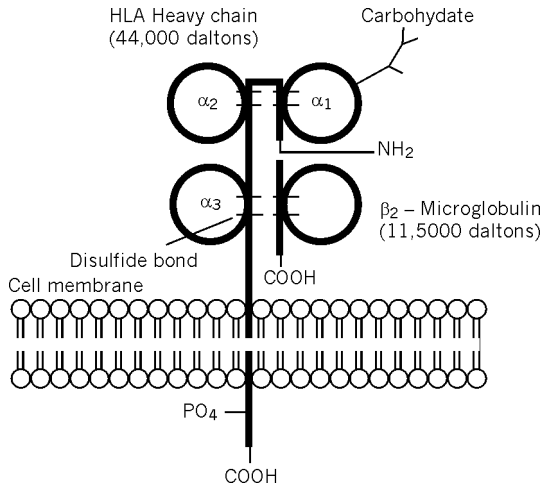


Figure 10.4. Structure of the MHC Class I antigen.

distal from the cell membrane and form a groove along the top surface of the molecule. The sides of the groove are formed by the α1 and α2 domains, the stretches of protein that form the walls of the groove lie in an alpha helix configuration. The base of the groove also contains parts of these α1 and α 2 domains, the protein stretches of the base are found in a β pleated configuration(Figure 10.5). Analysis of the protein stretches forming the groove show that there are hypervariable regions along the sides of the groove and to a lesser extent in the β pleated base. In all studies concerning the MHC Class I antigen, workers found that the groove always contained an unidentified peptide molecule, presumably representing processed antigen. These findings are consistent with the idea that MHC molecules bind and present processed antigens to responding T cells and that the T cell receptor co recognizes foreign antigen only if presented with the MHC Class I antigen. The genes that determine a Class I antigen consist of several exons interspaced by introns. A separate exon codes for each of the three α domains and gene polymorphism exists within each of these exons, more so in the α 1 and α 2 regions.

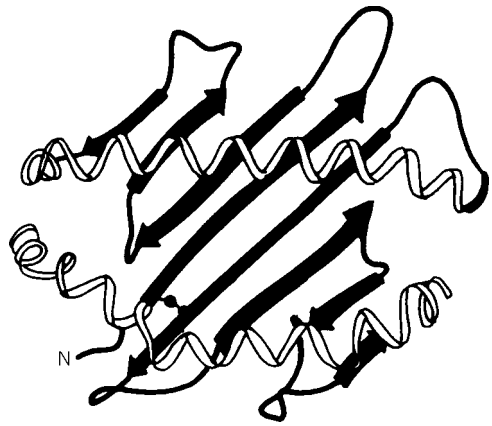


Figure 10.5. A schematic representation of the α2 and α3 domains of the MHC Class I antigen reveals a groove thought to be involved in antigen binding and presentation to T cells. The view is looking down on the top of the molecule, showing the surface that faces away from the cell membrane. The groove has a base composed of eight β strands, shown as thick arrows pointing in the amino to carboxyl direction. The helices, which form the sides, are shown as helical ribbons. The two connected spheres represent a disulfide bond, and the N terminus is indicated. Polymorphism is found both along the edges of the helices and in the base.

MHC Class I antigens are expressed on all cell types except erythrocytes and trophoblasts. Striated muscle cells and liver parenchymal cells are normally negative for Class I antigens or they may express only a low density of Class I molecules. However, in inflammatory states these cells begin to express large numbers of Class I molecules.

By a process called capping, it can be shown that the MHC Class I antigens are separate entities on the cell membrane. When exposed to specific antibody, the MHC antigen on the cell surface will move from its usual, even distribution, to a single small area or cap on the cell. If a cell is exposed to antibody to HLA–A antigen, only these antigens will form a cap; the HLA–B and HLA–C antigens will remain evenly distributed. If antibody to the β2 microglobulin is used, all antigens will cap as this molecule is common to all Class I antigens. The β2 microglobulin helps to keep the configuration of the MHC Class I antigens. If the β2 microglobulin is selectively removed, the structural configuration of the antigen is lost; particularly that of the groove between the α1 and α 2 domains.

Functions of the MHC Class I antigens

In cell mediated cytolysis the Class I antigens are the target antigens recognized by the killer/ cytotoxic T lymphocytes. MHC class I molecules specifically bind CD8 molecules expressed on cytotoxic T lymphocytes. Class I antigens are the principal antigens recognized by the host during tissue graft rejection.

The true physiologic role of the MHC Class I antigens lies in the cell mediated lysis of virus infected cells. When T lymphocytes are exposed to a viral antigen, they will recognize it only if associated with a Class I antigen; i.e., these viral antigens are presumably the peptides found within the groove formed between the $\alpha 1$ and $\alpha 2$ domains. The cytotoxic T lymphocytes elicited by such an exposure are restricted in their killing to those target cells which bear both the same viral antigen and the same Class I antigen as were present on the cell that first stimulated their proliferation. These T lymphocytes will not kill target cells bearing the same viral antigen and a different Class I antigen, nor will they kill cells bearing the correct Class I antigen and a different viral antigen.

In summary MHC class I expression is widespread on virtually every cell of the body. This is consistent with the protective function of cytotoxic T lymphocytes which continuously survey cell surfaces and kill cells harbouring metabolically active microorganisms. MHC class I molecules bind peptide fragments derived from proteolytically degraded proteins endogenously synthesized by a cell. Small peptides are transported into the endoplasmic reticulum where they associate with nascent MHC class I molecules before being routed through the Golgi apparatus and displayed on the surface for recognition by cytotoxic T lymphocytes.

MHC Class II antigens

Class II antigens are found chiefly on surfaces of immunocompetent cells, including monocytes/macrophages, activated T cells, dendritic cells and most notably on B cells. As with Class I antigens, inflammatory states cause many other tissues to express Class II antigens. In humans the Class II antigens are encoded by the HLA–D gene region, which is divided into at least three sub regions : HLA–DP,HLA–DQ and HLA–DR. The detailed structure of an HLA–DR molecule has been elucidated and serves as a prototype for the Class II molecule. The MHC Class II antigen, like the Class I antigen, consists of an α chain (34,000 daltons) and a β chain (29,000 daltons) in non covalent association (Figure 10.6). Both chains are anchored in the cell membrane and display an extracellular hydrophilic region, a transmembrane hydrophobic region and an intracellular hydrophilic region. The α chain intracellular region can be phosphorylated. As shown in figure 10.7 both chains contain extracellular domains termed $\alpha 1$, $\alpha 2$ and $\beta 1$, $\beta 2$. Positions of disulphide bonds and carbohydrate linkages are also illustrated in the figure. Like in the MHC Class I molecule, the sides of the $\alpha 1$ and $\beta 1$ domains form a groove in which the sides are in an α helix configuration and the base contains β pleats. Again, hypervariable regions are located primarily along the groove which suggests that foreign antigen rests within this groove and that T cells recognize foreign antigen in conjunction with the MHC antigen.

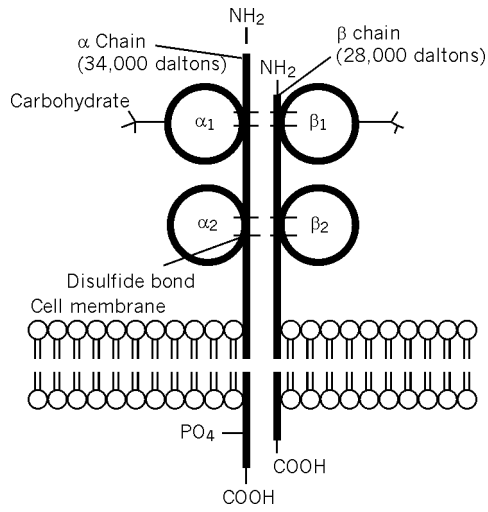


Fig. 10.6. Structure of the MHC Class II antigen.

The genetic organization of the MHC Class II antigens is a little more complex (Figure 10.7). α genes encode the α chains and β genes code for β chains. As shown in Figure 10.7, the HLA –DR gene region has one locus for the α chain and three loci for the β chain; although one of the latter may be a pseudogene (ie: it does not determine a product). The DR α chain can combine with any of the ß chains to produce a DR molecule. The same happens for the DP and DQ molecules.

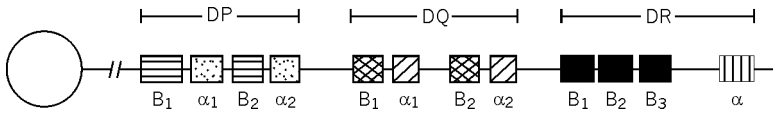


Figure 10.7. Genetic organization of the MHC Class II antigen showing the DP, DQ and DR regions and their constituent α and ß chain genes.

Functions of the MHC Class II antigens

The MHC Class II antigens are principally responsible for an in vivo correlate of the mixed lymphocyte reaction (MLR) –the graft versus host reaction. In the physiological situation foreign antigen is recognised in conjunction with Class II molecules by certain groups of T cells: the CD4+ T cells or T–helper cells. Hence MHC Class II molecules play an important part in presenting processed antigen to T cells and aid collaboration between T and B cells. Whether T cells recognize antigen in conjunction with Class I or Class II molecules, it follows that T cells must be equipped with receptors both for the MHC antigens as well as foreign antigens. This phenomenon, when T cell activity is restricted to those cells which bear antigen in conjunction with either the MHC Class I or Class II antigens, is known as the **MHC Restriction Phenomenon**.

In summary, MHC class II expression is restricted to "antigen presenting cells." This is consistent with the functions of helper T_H lymphocytes which are locally activated wherever these cells encounter macrophages, dendritic cells, or B cells that have internalized and processed antigens produced by pathogenic organisms. MHC class II molecules bind peptide fragments derived from proteolytically degraded proteins exogenously internalized by "antigen presenting cells," including macrophages, dendritic cells, and B cells. The resulting peptide fragments are compartmentalized in the endosome where they associate with MHC class II molecules before being routed to the cell surface for recognition by helper T lymphocytes.

Disease and the Major Histocompatibility Complex

Diseases associated with HLA antigens have several characteristics : (a) they are of unknown cause and unknown pathophysiologic mechanism, with a hereditary pattern of distribution, (b) they are associated with immunologic abnormalities and (c) they have little or no effect on reproduction. Table 10.1 lists the diseases showing positive HLA - disease associations.

Table 10.1: Diseases with positive HLA associations

Disease		HLA Antigen	Relative Risk
Rheumatic	Ankylosing spondylitis	B27	69.1
	Reiter's syndrome	B27	37.0
	Acute anterior uveitis	B27	8.2
	Reactive arthritis (yersinia, salmonella, campylobacter)	B27	18.0
	Psoriatic arthritis (cenral)	B27 B28	10.7 9.1
	Psoariatic arthritis (peripheral)	B 27 B38	2.0 6.5
	Juvenile rheumatoid arthritis	B27	3.9
	Juvenile rheumatoid arthritis pauciarticular	DR5	3.3
	Rheumatoid arthritis	DR4	3.8
	Sjogren's sundrome	DR3	5.7
	Systemic lupus erythematosus	DR2 / DR3	2.6
Gastro-intestinal	Gluten sensitive enteropathy	DR3	11.6
	Chronic active hepatitis	DR3	6.8
	Ulcerative colitis	B5	3.8
Haematologic	Idiopathic haemochromatosis	A3 B14 A3, B14	6.7 26.7 90.0
	Pernicious anaemia	DR5	5.4
Skin	Dermatitis herpetiformis	DR3	17.3
	Psoriasis vulgaris	B13, B17, Bw57, Cw6	7.5
	Behcet's disease	B5	3.8
Endocrine	Insulin dependent diabetes mellitus	DR4 DR3 DR2	3.6 4.8 0.2
	Graves disease	B8 DR3	2.5 3.7
	Addison's disease	Dw3	10.5
	Subacute thyroiditis (deQuervain)	B35	13.7
	Hashimoto's throiditis	DR5	3.2
	Congenital adrenal hyperplasia	Bw47	15.4

Disease		*HLA Antigen*	*Relative Risk*
Neurologic	Myasthenia gravis (without thymoma)	B8	3.3
	Multiple sclerosis	DR2	2.7
	Bipolar affective disorder	B16	2.3
	Narcolepsy	DR2	130.0
	Schizophrenia	A28	2.3
Renal	Idiopathic membranous glomerulonephritis	DR3	5.7
	Goodpasture's syndrome	DR2	15.9
	Minimal change nephrotic syndrome	DR7	4.2
	IgA nephropathy	DR4	3.1
	Polycystic kidney disease	B5	2.6
Infectious	Tuberculoid leprosy (Indians)	B8	6.8
	Paralytic polio	B16	4.3
	Low vs high response to vaccinia virus	Cw3	12.7

The prototype HLA –disease association is that of ankylosing spondylitis with **HLA–B27**. Ninety per cent of American Caucasian patients with ankylosing spondylitis possess HLA–B27. In some cases a disease may be associated with antigens determined by 2 different HLA loci (refer Table 10.1).

Several hypotheses have been advanced to explain HLA disease associations. In the 1960's, it was discovered that the mouse MHC (called H2) played a part in the genetic susceptibility of mice to certain leukaemias and in their immune response to certain antigens. Since then numerous reports have been published describing the role of the human MHC in the control of immune responsiveness and disease susceptibility.

There are two general explanations for HLA and disease associations. Firstly, there may be linkage disequilibrium between alleles at a particular disease associated locus and the HLA antigen associated with that disease: this is so for HLA–A3 and idiopathic haemochromatosis.

Another possible explanation is that the HLA antigen itself plays a role in disease; as explained by one of the following models:

(*a*) by being a poor presenter of a certain viral or bacterial antigens

(*b*) by providing a binding site on the surface of the cell for a disease provoking virus or bacterium

(*c*) by providing a possible means of transport for the virus to enter the cell

(*d*) by having close molecular similarity to the pathogen, the immune system fails to recognise the pathogen as foreign and fails to mount an immune response against it.

It is most likely more than one mechanism is involved, but to varying extents in different diseases. In multiple sclerosis and ankylosing spondylitis, cell mediated immunity is often depressed, not only in the patients but also in their parents and siblings. Complement (C2) levels are known to be low in systemic lupus erythematosus, a disease associated with HLA DR2 and DR3. In gluten enteropathy, which shows a high association with HLA DR3, a specific gene product is thought to act as an abnormal receptor for gliadin, the wheat protein, and present it as an imunogen to the body.

Whatever the explanation for HLA and disease association, it is clear that the HLA system and other non-linked genes operate concurrently to produce disease.

CHAPTER – 11

IMMUNE RESPONSE MECHANISMS I: B AND T LYMPHOCYTES

The Origin of B Lymphocytes

The B lymphocyte and its secreted end product, the antibody molecule has been recognized as a powerful immunologic tool for over a century. Studies in birds showed that the bursa of Fabricius, a hindgut lymphoid organ was the site of early development of antibody producing cells. This lineage of cells was therefore termed B cells. In mammals, cells of B lineage are initially generated in the foetal liver, when haemopoietic stem cells migrate to the liver from the yolk sac. This process begins during the 8th week of human gestation. The foetal liver continues to be a major site for production of the erythroid/ myeloid series including B cells, until well into the second trimester. Stem cells then populate the bone marrow, thereafter B cells are continuously produced in the bone marrow throughout life. B cells develop from a pluripotential stem cell that can give rise to all of the different types of haemopoietic cell types.

The Biology of B Lymphocytes

B lymphocytes display immunoglobulins as integral proteins of their cell membranes. These membrane bound immunoglobulins are the B cell receptors for antigen and they differ from secreted immunoglobulins in several ways. They are anchored in the cell membrane with the carboxy terminal embedded into the membrane. The trans membrane region is hydrophobic in nature. The amino or antigen binding end is extracellular and strategically placed to receive foreign antigen. Immature B cells initially display membrane bound IgM monomers. IgD molecules are not found on newly formed B cells. Immature B cells respond negatively to cross linkage of their IgM molecules by multivalent antigen. Thus early exposure to antigen eliminates B cell responsiveness leading to tolerance towards that antigen. As B cell development proceeds, IgD molecules appear on their surface membranes along with IgM and the IgD becomes the predominant membrane bound isotype found. Resting B cells display higher levels of surface IgD; as activation of B cells commences IgD is lost and other receptors that aid B cell activation appear on the cell surface.

Immature B cells rapidly acquire receptors for C3d, Epstein - Barr virus, C3b and Fc portions of IgG molecules. Histocompatibility Class I antigens are present on immature B lymphocytes. Activated B cells express increased amounts of HLA-D region encoded molecules. Receptors for Fc(gamma) and C3b also show an increase. The latter two then decrease during the terminal stages of B cell differentiation. Activated B cells also display receptors for interleukin-2 (IL-2) and other factors needed for full differentiation of B cells. Figure 11.1 shows the full complement of receptor molecules on the B cell surface. Monoclonal antibodies are now available which can identify cell surface markers and thereby accurately stage the B cell in its development.

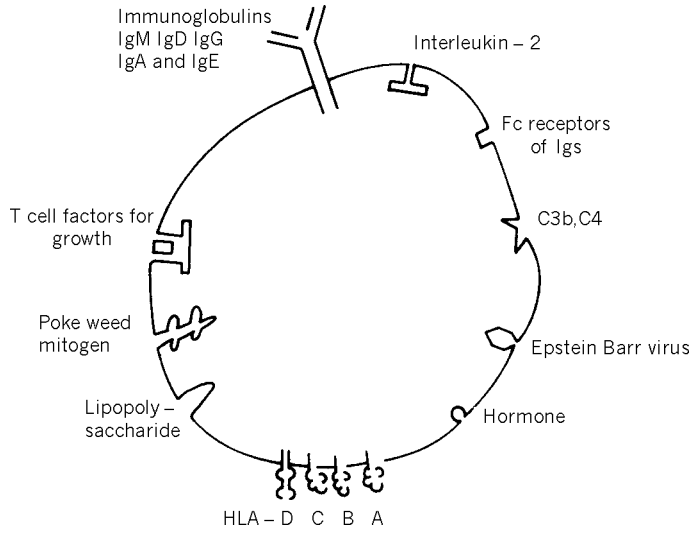


Figure 11.1. Receptor molecules on the B Cell surface.

After antigen or mitogen stimulation B cells can proceed along one of two pathways. They can differentiate into plasma cells and secrete large amounts of immunoglobulin or they can divide and return to a resting stage. These latter cells are called memory B cells and can rapidly differentiate into plasma cells following second exposure to the same antigen. Plasma cells are the terminally differentiated state of B lymphocytes. The antibody forming machinery of these cells are turned up full force. Over 40% of the total proteins produced by plasma cells are immunoglobulins.They release thousands of antibody molecules every second. The plasma cell seldom divides and has an average life span of less than 4 days.

The Clonal Selection Theory

How do cells make antibodies in such enormous variety? One antibody can neutralize just one type of antigen - and antigens come in an incredible diversity of shapes, sizes and chemical compositions. Yet, the human system is capable of manufacturing a practically unlimited range of antibodies against bacteria and their toxins, viruses, pollen grains, incompatible blood cells and even some novel man made molecules.

Over 30 years of intensive research has led to the doctrine that is most widely accepted today and is called the **Clonal Selection Theory** of antibody formation. The conceptual father of the clonal selection theory was **Paul Ehrlich**. Over a hundred years ago he postulated that a white blood cell's surface bore receptors with side chains to which foreign substances became chemically linked. This binding prompted the cell to produce and secrete copies of this bound receptor - the antibody -into the circulation (Figure 11.2). For decades after Paul Ehrlich proposed his theory, he failed to win validation in scientific circles. It was only in the 1960s that immunologists resumed their search and brought this theory into its own.

It was **Niels Jerne** who introduced the concept of clonality and formulated a notion similar to Ehrlich's side chain theory. He suggested that any animal possesses in its armamentarium, small numbers of preformed antibodies against all antigens. An antigen–antibody complex interacts with white cells and more antibody to the same antigen is produced. **David Talmage**

in 1957 went a bit further, to say that white cells are selected for proliferation when the antibody they synthesize matches the invading antigen.

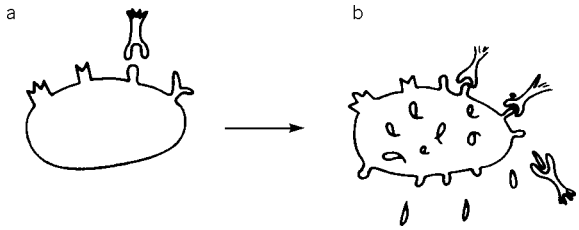


Fig.ure 11.2. Ehrlich's side chain theory (a) Combination of antigen with preformed receptors (b) Cell is triggered to produce and secrete more of these receptors.

In retrospect it is obvious that Jerne and Talmage laid the foundations of the clonal selection theory , it remained for **Macfarlane Burnet** to crystallize the concept of clonal selection. Central to Burnet's thinking was the tenet that one cell produced just one kind of antibody. He proposed that binding of an antigen with an antibody -cum-receptor on the cell surface, triggers the cell to multiply and manufacture more of the same receptor. He asserted that each cell and its clones, or offspring, can produce just one kind of antibody and he coined the term clonal selection to describe his theory. Figure 11.3 illustrates the clonal selection concept.

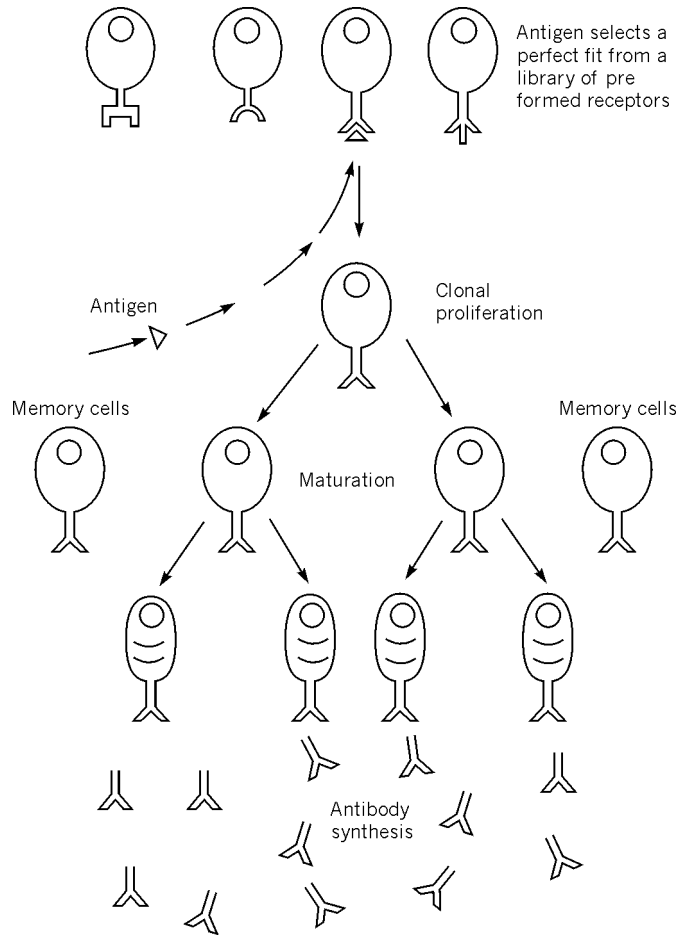


Figure 11.3. The Clonal Selection Theory.

The human system is endowed with antibody producing cells bearing an entire library of antigen binding receptors (the immunoglobulin molecule). This occurs during development; each B-lymphocyte becomes genetically programmed, through a process called **gene translocation**, to make a unique B-cell receptor. Molecules of that B-cell receptor are placed on its surface where it can react with epitopes of an antigen. If an antigen enters the system it matches with one of the receptors and binds to it. This induces the cell to proliferate, to produce several clones of its kind, each capable of secreting more of just that one particular type of receptor (antibody) matched for the invading antigen. Once an entire cadre of cells has been generated, the second response to the same antigen is more intense since there are many more cells awaiting to bind antigen. Each cell will then generate more clones secreting the same antibody. Once clones of antibody producing cells have been generated, prolonged exposure to antigen is not necessary to maintain antibody production. The clonal selection theory together with an understanding of the genetics of antibody diversity has provided many answers regarding the mechanisms which control the production of the vast repertoire of antibodies that the immune system is endowed with.

Finally, clonal selection explained immunologic tolerance as the complete deletion of an entire clone of cells, which would occur before or after birth, if an antigen overwhelmed the metabolic capabilities of the cells. In 1960, Macfarlane Burnet shared the Nobel Prize with Peter Medawar for his accomplishments in understanding immunologic tolerance. In 1984, Niels Jerne also received the Nobel Prize for his theoretical contributions in conceptualizing the idea of clonality in immunology.

T-Lymphocytes

The thymus derived lymphocytes, or T cells mediate 2 general types of immunologic functions: effector and regulatory. The effector functions are mediated by (*i*) secretion of soluble substances called lymphokines or cytokines and (*ii*) by their ability to kill other cells (cytotoxicity). The regulatory functions are represented by their ability to amplify cell mediated cytotoxicity by other T cells and by the "help" they render in the production of immunoglobulins by B cells. These functions also require the synthesis of lymphokines (cytokines).

Stem cells migrate to the thymus and move from cortex to medulla and out into the periphery - a journey that takes three days. As T cells gain maturity, certain surface molecules begin to be expressed. An understanding of T cell function has been possible because of the identification and characterization of these surface molecules. The most important of these surface molecules is listed in Table 11.1.

Table 11.1: Molecules on the T cell surface

T cells subsets	*Main cell surface markers*	*Restrictions*	*Functions*
T helper cell or T4 cell	CD4+; CD3–T–cell receptor; CD2+	MHC–Class II	Stimulate B cell to produce antibody; Induce CD8+ T–cell cytokine secretion; macrophage activation
T cytotoxic cell or T8 cell	CD8+; CD3–T–cell receptor; CD2+	MHC–Class I	Lyse antigen bearing target cell

Each of the molecules listed is detectable by monoclonal antibodies and plays an important role in T cell differentiation and function. As shown in Figure 3.2, stem cells do not display any surface molecules. As T cells move into the thymic cortex, the CD2 molecule is the first to appear, followed by CD3, CD4 and CD8. In the thymic medulla, two distinct lineages become evident: one bearing CD2, CD3 and CD4 molecules and the other bearing CD2, CD3 and CD8 molecules. In peripheral lymphoid tissue, 65% of the cells possess CD2, CD3 and CD4 molecules on their surface and 35% display CD2, CD3 and CD8 antigens.

CD4+ T cells or T4 cells constitute the helper T cells and CD8+ T cells or T8 cells form the cytotoxic/suppressor T cells. This demarcation, however, is not water tight and it has been shown that T4 cells take part in cytotoxicity targeted at cells bearing MHC Class II molecules. Broadly, the T helper or T4 cells, render B cell help, they secrete cytokines to propagate this function and also to amplify cell mediated immunologic reactions. The T cytotoxic (T8) cells act as specific killer cells.

In the thymus all T cells learn to recognize self MHC gene products and this helps them to react with antigen in conjunction with MHC gene products in the periphery.

T4-Lymphocytes (T4-Helper Cells, CD4$^+$ Cells)

Functionally, there are different types of T4-lymphocytes based on the cytokines they produce. The two primary types are T_h1 cells and T_h2 cells. (Both T4-lymphocytes and T8–lymphocytes can exhibit T_h1 or T_h2 cytokine profiles).

T_h1 lymphocytes

T_h1–lymphocytes recognize antigens presented by macrophages and function primarily to activate and heighten cell-mediated immunity by producing cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN-gamma) and tumour necrosis factor-beta (TNF-beta). Collectively these cytokines enable T8-lymphocytes to proliferate and differentiate into cytotoxic T–lymphocytes capable of destroying infected host cells and mutant cells; activate cytotoxic T–lymphocytes and NK cells; activate macrophages enabling them to destroy intracellular pathogens; stimulate the production of opsonizing and complement-activating antibodies for enhanced attachment during phagocytosis; activate neutrophils; stimulate increased production of monocytes in the bone marrow; and function as chemoattractants for phagocytes.

T_h2 lymphocytes

T_h2-lymphocytes recognize antigens presented by B-lymphocytes. They produce cytokines such as interleukins 2, 4, 5, 10, and 13 that promote antibody production. Collectively these cytokines enable activated B-lymphocytes to proliferate, stimulate activated B–lymphocytes to synthesize and secrete antibodies, promote the differentiation of B-lymphocytes into antibody–secreting plasma cells, and enable antibody producing cells to switch the class of antibodies being produced. Another major function of the cytokines produced by T_h2 cells is to enable B–lymphocytes to activate eosinophils and produce increased amounts of IgE against helminths. IgE acts as an opsonizing antibody enabling eosinophils to attach to helminths for extracellular killing.

T8-Lymphocytes (T8-Cells; CD8$^+$Cells; Cytotoxic T-Lymphocytes)

Cytotoxic T cells consititute one of the body's major defences against viruses, intracellular bacteria, and tumour cells. These cytotoxic T cells are effector cells derived from T8-lymphocytes

during cell-mediated immunity. However, in order to become cytotoxic, naive T8-lymphocytes must become **activated** by cytokines produced by antigen-presenting cells (APCs). This interaction between APCs and naive T8-lymphocytes occurs primarily in the lymph nodes, the lymph nodules, and the spleen.

The T Cell Receptor for Antigen

Few problems in immunology have been as difficult to solve and as controversial as that of characterizing the T cell receptor for antigen. The B cell receptor for antigen has long been identified as the IgM monomer bound on its surface. Further, the B cell receptor is capable of recognizing and reacting against free, native antigen. Studies in the 1960s and early 1970s clearly demonstrated that T cells too, were antigen specific. Therefore it followed that T cells must have a receptor capable of recognizing antigen. The molecules comprising the T cell receptor and the genes that code for them have been characterized.

The T cell receptor is a heterodimer composed of 2 chains, each of molecular weight 40-50,000 daltons, linked by a disulphide bond on the T cell surface (Figure 11.4).The two chains are termed α and β chains and are integral membrane proteins extending 4-5 amino acids into the cytoplasm. Each chain is folded into two domains, one having a relatively constant structure, much like the constant region of the immunoglobulin molecule. The other domain exhibits far more variability than the immunoglobulin molecules and is analogous to the immunoglobulin variable region. It stands to reason, therefore, that the variable regions are strategically placed and constructed to bind with foreign antigen.

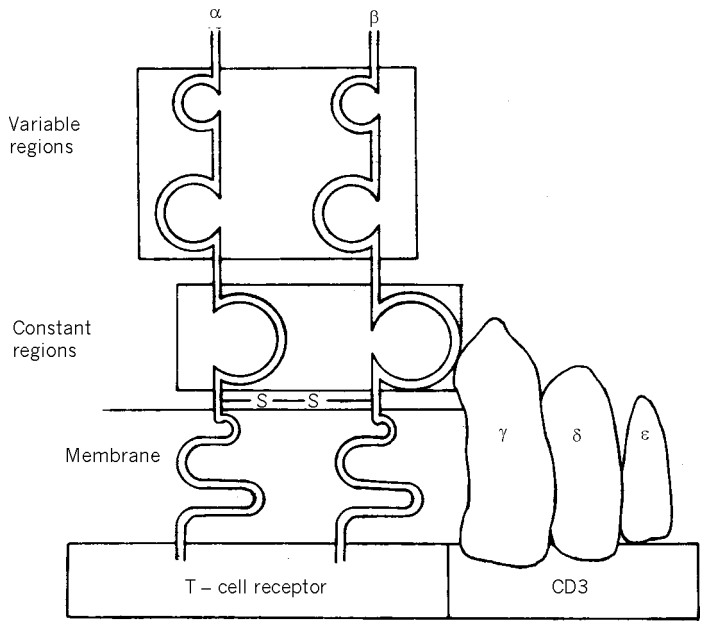


Figure 11.4. The *T* cell receptor for antibgen.

Genetic Organization of the T Cell Receptor

A study of the genetic organization of the T cell receptor (TCR) led to the understanding that T cells too, are capable of recognizing an enormous range of antigens. Separate sets of genes encode the α and β chains. The genetic locus containing the α chain genes is present on chromosome no:14, while the genes coding for the β chain are present on the 7th chromosome. These genetic loci can be further divided into regions similar to the gene arrangement for the immunoglobulin molecule (refer Chapter 6). The regions have been named: the variable region genes (V), diversity segment genes (D), joining region regions (J) and constant region regions (C). Each gene region is comprised of gene clusters, for example, there are 60 V region genes for the α chain and 21 for the β chain. Other regions also, are highly polymorphic and have several gene clusters. The polymorphic genetic organization of the T cell receptor is shown in Table 11.2.

Table 11.2: Genetic organization of the T cell receptor

Gene regions	*α chain*	*β chain*
V region	60	21
D region	–	2
J region	40	12
C region	1	2

Rearrangements occur between gene segments, analogous to that described for the immunoglobulin molecule (refer Chapter:6). This provides the diversity to account for the existence of over a million distinct T cell clones per individual, each having a different antigen specificity. Antigen diversity is accounted for not only by rearrangement of gene segments as with the immunoglobulin molecule, but also by interactions between the α and β chains of the T cell receptor molecule. Therefore, different antigen specificities are created when a single α chain interacts with different β chains.

The CD3 Complex

In all immunocompetent T cells the T cell antigen receptor is non covalently but intimately linked with closely related molecules called the CD3 complex. The three peptide molecules of the CD3 complex have been called CD3/γ CD3/δ and CD3/ε (Figure: 11.4). Each of the CD3 molecules is an integral part of the T cell membrane and extends into the cytoplasm farther than either the α or β chains of the receptor. The CD3 molecules transduce the antigen recognition signal received by the α – β heterodimer to the inside of the cell.

Surface CD4 and CD8 Molecules

The CD4 and CD8 molecules play an important role in T cell activation. Both the CD4 and CD8 molecules and the genes that encode them have been identified. There is no evidence of polymorphism or gene rearrangement in the case of these molecules and they appear identical in cells having different antigen specificities. Therefore they do not seem to participate in antigen recognition.

The CD4 and CD8 molecules have been shown to function as recognition markers for the MHC gene products. The presence of CD4 on T cells indicates that the T cell is programmed to recognize and interact with cells bearing MHC class II molecules; while T cells bearing CD8 molecules recognize cells displaying MHC Class I molecules as part of their antigen specificity. It has been hypothesized that the CD4 and CD8 molecules are not involved in activation of T cells, but can increase interaction between T cells and target cells. They seem to behave like extra "glue", so that activation can occur. For cells seeing antigen for the first time this "glue" may play an important role. It is also thought that since they are structurally constant, they play a part in recognizing the constant regions of the MHC Class I and II molecules. This is borne out by the finding that the CD4 and CD8 molecules are closely associated with the T cell receptor and actually bind to the constant region of the MHC Class I and II molecules, thus guiding the T cell receptor onto the antigen (Figure 12.2, refer Chapter 12).

The CD2 Molecule

The CD2 molecule on T lymphocytes is a transmembrane surface glycoprotein which facilitates cell-cell contact. The amino terminal domain of CD2 (domain 1) mediates its adhesion function by binding to LFA-3 (CD58), another cell surface glycoprotein widely expressed on various cell types including hematopoietic and epithelial cells. Both CD2 and CD58 are members of the **Immunoglobulin gene superfamily (see below)**. The importance of CD2 function in the normal human immune response has been well documented: (*i*) recognition involving helper T cells and antigen presenting cells; and (*ii*) for the cytolytic effector function of natural killer (NK) cells and cytotoxic T lymphocytes (CTL).

The Immunoglobulin Gene Superfamily

All the molecules involved in antigen recognition described so far are members of, what is now known as, a "gene superfamily". These are the heavy and light chains of the immunoglobulin molecule, the α and β chains of the T cell receptor, the α and β 2 microglobulin chain of the MHC Class I antigen and the α and β chains of the MHC Class II antigen (Figure 11.5). It is obvious that all of these molecules have certain common characteristics ; they all recognize and bind foreign antigen and to do this they are equipped with variable regions as part of their structures. This is consequent to the polymorphic gene structure and characteristic gene rearrangements seen in all of these molecules. It has been found that certain sequences (about 110 amino acids long) are common to all three structures. Besides these, homologous sequences have been conserved around disulphide bonds and in the characteristic antiparallel β pleated strands. This characteristic β pleated folding of short protein stretches is a shared feature between all three molecules. An additional feature, common to these structures, are that many are 2 chain molecules with strong interdomain non covalent interactions. It is believed that all three molecules evolved from a single primordial ancestral gene. The success of these molecules indicate that the forces of evolution will make sure that they are widely exploited in nature. Since molecules unrelated to the immune system also fulfil criteria for inclusion into the "super gene family", it has been suggested that recognition of cell surface molecules (immune or otherwise), is the primary role of these molecules.

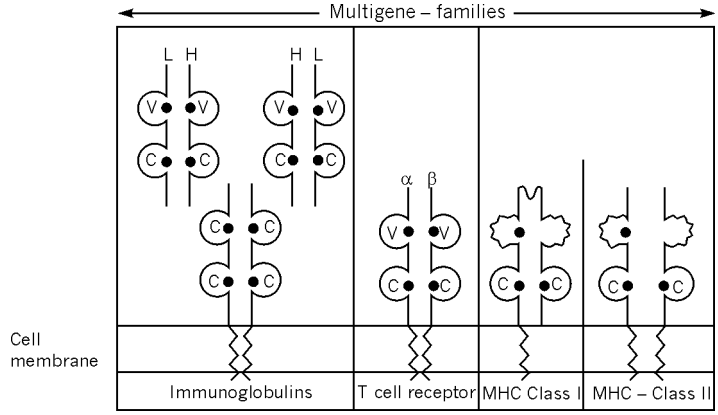


Figure 11.5. The Immunoglobulin gene superfamily

Regulatory (Suppressor) T cells

The existence of specific suppressor T cells and the phenomenon of immunologic suppression has fallen into disfavour in the present decade. However, most immunologists believe that some form of suppression exists. The reason for the suppressor cell controversy is that no one has been able to clone a unique suppressor T cell. Of the original suppressor T cells (Ts cells), the most promising recent candidates have been given other names. Examples of these are:

- Tr cells
- Tr1 cells
- Th3 cells

Tr Cells

Most CD4+ T cells belong to either the T_h1 or T_h2 subsets. However some 5–10% of them do not. These have been termed T-regulatory (Tr) cells. Like other T cells their cell surface molecules have been characterised. They express a transmembrane protein called **CD25**, which is the α chain of the receptor for interleukin-2 (IL-2). Like other T cells, they also express the α β (alpha-beta) T-cell receptor for antigen (TCR) and can only be activated if it binds to the peptide-class II MHC molecule. If activated, they secrete large amounts of **interleukin 10 (IL-10)** and often some transforming growth factor-beta (**TGF-β**) as well. Both these lymphokines are powerful **immunosuppressants** inhibiting both **T_h1** help for cell-mediated immunity (including graft-versus-host disease) and inflammation, and **T_h2** help for antibody production, and, possibly, the action of CD8+ cytolytic T lymphocytes (CTL).

The antigenic peptides recognized by their TCRs tend to be **self**-peptides and perhaps the major function of Tr cells is to inhibit other T cells from mounting an immune attack against self components; that is, to protect the body against **autoimmunity**.

Tr1 Cells

Tr1 cells resemble Tr cells in several ways, although they do not express large amounts of CD25 on their surface. They require IL-10 for their formation and once mature, secrete large amounts of it. Tr1 cells are abundant in the intestine, and their chief function may be to make

the host tolerant to the many antigens that are part of its diet. It is believed that patients who develop Crohn's disease lack the down regulation characterised by IL-10 via the Tr1 cells making them intolerant to a number of dietary and other antigens.

Th3 Cells

Th3 cells are also prevalent in the intestine, but unlike Tr1 cells, their main lymphokine is TGF-β. Also like Tr1 cells, they suppress immune responses to ingested antigens. Further research is needed to elaborate the relationships between these and other T cells that suppress immune responses. This work is relevant for patients who risk rejection of organ transplants; and for the management of patients with autoimmune disorders like lupus erythematosus, insulin-dependent diabetes mellitus (IDDM-type I diabetes) and Crohn's disease.

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CHAPTER – 12

IMMUNE RESPONSE MECHANISMS II: ANTIGEN PRESENTATION AND PROCESSING; MECHANISMS OF LYMPHOCYTE ACTIVATION

When cells of the immune system encounter an antigen, a humoral immune response or cellular immune response, or both may occur. Humoral immunity is mediated by B cells, which after stimulation proliferate and differentiate into antibody producing plasma cells. Cellular immunity is mediated by T cells, which become activated to secrete a number of substances important in the immune response; they also kill virus infected and malignant cells directly, fulfilling the role of T cytotoxic lymphocyte(Tc/CTL) or killer T cells. Enhancing and supplementing the activity of B cells, are another set of T cells. These T cells help B cells to proliferate and secrete antibodies and are popularly known as T helper (Th) cells. It is therefore obvious that T cells and B cells need to communicate and interact with each other and with other antigen presenting cells. This they do through receptors, soluble factors and various cell adhesion molecules.

Antigen Presentation

Very early studies have shown that T cells and B cells recognize different parts of an antigen. If a hapten is coupled with a carrier particle to yield an immunogen, B cells react with the hapten to produce hapten specific antibodies whereas T cells respond predominantly to parts of the carrier molecule. Presentation of antigen to T cells differs from presentation of antigen to B cells. B cells can recognize native, unmodified antigen and this antigen need not be presented in conjunction with cellular MHC antigens. Though most B cells do require T cell help for an adequate antibody response and for memory cells to be generated.

In contrast, T cells do not recognize native antigen; antigen must first be **processed** by an antigen presenting cell and then **presented** in association with MHC Class I or Class II molecules. In other words, T cells are blind to native antigen and will "see" antigen only when processed and presented together with an MHC molecule. To reiterate a general rule of thumb that has been discussed in previous chapters: the CD8+ T cell recognizes antigen in association with an MHC Class I molecule and a CD4+ T cell recognizes antigen in conjunction with an MHC Class II molecule. More often than not, CD4+ T cells act as helper cells and CD8+ T cells act as cytotoxic cells, though this is not a rigid rule as we shall see in the following sections.

The Pathways of Antigen Processing

Macrophages were the first accessory or antigen presenting cells to be identified. Later dendritic cells (specialized cells found in the lymph nodes and spleen) and B cells themselves were added to the principal list of potential antigen presenting cells. Several antigen presenting cells (APCs) do more than simply capture antigen and display it on their surface, they break

down antigen into peptide fragments before presenting it, obscuring its shape and leaving only its distinctive amino acid sequence as the target - a process that requires both time and energy. This distinctive target peptide fragment processed within the APC, is then transported to the surface of the cell and displayed in the antigen binding cleft or groove that is an integral part of both MHC Class I and Class II molecules (Refer chapter 10). This explains why investigators found that T cells have no interest in native antigen shape.

A scheme that describes the antigen processing pathway takes into account all of the above data and is illustrated in Figure 12.1. **Pathway A,** describes the events that take place when the antigen concerned is freely circulating foreign material (not found intra cellular or bound to cell surfaces). Such an antigen has been termed "exogenous" antigen and is taken up by specific classes of APCs such as B cells, macrophages or dendritic cells that are specialized for processing exogenous antigen. The uptake of these antigens is either by receptor mediated endocytosis (which may be purely fortuitous), or as a result of simple pinocytosis. The antigen now lies in an endosome within the cytosol of the cell. In the endosome, under the influence of an acid pH milieu, cathepsin - like proteases denature or fragment the original protein molecule. The peptide fragments then bind within the cleft of the MHC Class II molecules which are also found in the cytosol of the cell. The MHC Class II – antigen complex is then transported to the cell surface in vesicles and displayed to the exterior. Such an MHC Class II–antigen complex is recognized preferentially by the CD4+ T cell– and T cell help for antibody production is on its way! (Discussed in the following section). The remaining peptides of the original protein antigen go into a lysosomal compartment for complete degradation.

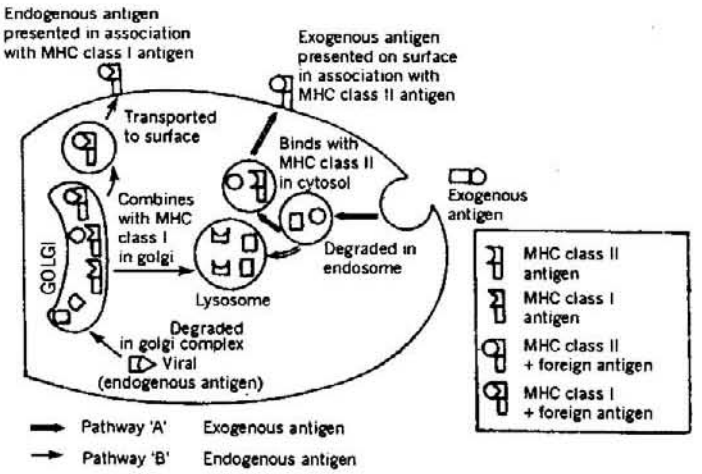


Figure 12.1 Pathways of antigen processing.

For those proteins that are borne as a result of **intracellular (such as viral) infection or malignant change** on a cell the pathway of processing is different (**Pathway B,** Figure 12.1). These antigens are known as "**endogenous**" antigens, being internal to the body's own cells. Processing of "endogenous" antigens occurs in the Golgi complex, an area specialized for dealing with improperly folded proteins synthesized within the cell (Figure 12.1). Such internally synthesized proteins need to be presented to the second major class of T cells, the cytotoxic T cells. Endogenous antigen is denatured or fragmented in the Golgi compartment, where they come into contact with MHC Class I molecules. The target peptide fragment binds into the

antigen binding groove on MHC Class I molecules and is transported to the exterior of the cell membrane. Such a peptide fragment bound to the MHC Class I molecule is preferentially recognized by the cytotoxic T cell - and the killer cytotoxic T cell has, therefore, been targeted onto the aberrant cell.

However, some proteins encoded by the genes of an infecting virus are synthesized in the **cytosol**. How does the cell get them into the Golgi compartment? This is achieved by specialist transport proteins called **TAP** (transporter associated with antigen processing) proteins. Viral proteins in the cytosol are degraded by proteasomes into viral peptides. The peptides are picked up by TAP proteins embedded in the membrane of the endoplasmic reticulum. Using the energy of ATP, the peptides are pumped into the lumen of the endoplasmic reticulum where they assemble with MHC class I molecule as described above. This complex then moves through the Golgi apparatus and is transported to the exterior of the cell membrane. Such a peptide fragment bound to the MHC Class I molecule is also preferentially recognized by the cytotoxic T cell.

Alain Townsend in 1985, showed conclusively that fragments of internal antigen, the viral nucleoprotein, were the antigens that were "seen" by cytotoxic T cells and these internal antigens initiated T cell cytotoxicity against virally infected cells. He had experimental evidence to show that whole surface antigens, therefore, could not be recognized by cytotoxic T cells– unprocessed as they were. Fragments of viral genes (the internal, nucleoprotein antigens), after being degraded and processed were displayed in a suitable manner - fitted into the MHC Class I-peptide binding groove. The antigen was then recognizable by killer T cells. These pioneering experiments helped evolve the current thinking regarding killing of virally infected or malignant cells by cytotoxic T cells. Credit also goes to Baruj Benacerraf and Hugh McDevitt who, in 1960, showed that genes of the MHC affected an animal's ability to mount an immune response to certain antigens; and to Don C.Wiley (1987), for elucidating the antigen binding cleft on the MHC molecule. The most striking feature of his work was the finding that this antigen binding cleft was not, and has never been found empty; in it Wiley and his colleagues found foreign peptide fragments - and presumed rightly that this was processed antigen.

Selection of peptides for surface expression probably depends on what fits most closely into the antigen binding groove of the MHC Class I and Class II molecules. Because exogenous antigen evokes CD4+ T cell help, it preferentially binds to MHC Class II antigens ; whereas, endogenous antigen needs to attract the attention of a cytotoxic T cell, it preferentially binds to the MHC Class I molecule: class discrimination in immunology!

Why does T cell recognition have to be so elaborate?

Why do T cells not recognize antigen directly as B cells do? Why do antigens need to be broken down and presented with MHC molecules? Alain Townsend and his co-workers, showed that a cell cannot even assemble Class I MHC molecules properly, unless a peptide is present during the final stages of the protein folding process. It seems that these omnipresent peptides are fragments of the body's own proteins and should the need arise they are displaced by foreign antigen peptides. There are several reasons put forth for this complex chain of events. For one, this scheme is consistent with the role of immune surveillance that T cells play. Killer T cells are known to constantly monitor the other cells of the body for the appearance of tumour or viral antigens and promptly eliminate any cell expressing them. By continuously processing and presenting their own antigens, cells invite inspection by the immune system so as to detect any early aberration. In the case of virally infected cells, MHC restricting guides a killer T cell to the culprit cell - the cell that is essentially the factory or manufacturing unit for thousands of virus particles. It ensures that a foreign organism cannot elude it by being hidden

inside a cell and adopting a Trojan horse strategy. Hidden antigens can also be processed and made visible! If, however, cells were to recognize freely circulating antigens, they would all be utilised by viral particles released by the manufacturing units and not be available to home in on renegade cells harbouring virus synthesis machinery. For the equally complex scheme of antigen presentation for helper T cells, the answer may be in the theory of evolution. Cell - mediated immunity appears to be a defence mechanism much older than man; even primitive sponges are endowed with this ability. Hence T cells may have originated as killer cells and may have acquired helper properties later in evolution. Therefore, the helper role seems to have superimposed itself into cells already programmed to recognize MHC molecules. Over the course of evolution, they have adapted this function to guide them to a site where they can be most effective to B cells which is, after all, the primary target of T cell help.

Understanding the phenomenon of antigen processing has helped investigators manipulate the immune system for clinical purposes. Traditionally, vaccines have consisted of whole pathogenic organisms, live or killed or a protein extract such as a toxin. For some diseases such as malaria this is not feasible, besides whole cell vaccines may have risky side effects. Vaccine scientists are now trying to design synthetic peptides representing a small fraction of the actual antigen, to trigger an immune response. To do so these peptides must stimulate both helper and cytotoxic T cells and bind to MHC molecules. MHC based technology should also make it possible to develop compounds that bind very strongly to those MHC molecules that bind self antigens and cause disease, as in autoimmune related disease states. By blocking the binding of self antigens on MHC molecules, such compounds might suppress the autoimmune response. Hence, some of the immune system's own precision and power can be directed to the fight against disease.

Mechanisms of Lymphocyte Activation

T cell activation

The first event that triggers T cell activation is T cell binding to antigen presented in conjunction with MHC molecules. The antigen binding cleft on MHC molecules shows variability, thus supporting the theory that they were made to bind foreign peptides. The T cell receptor /CD3 complex, therefore interacts with foreign peptide. Simultaneously the regions of the MHC molecule that display this antigen, on the surface of the APC interact with the appropriate CD4 or CD8 molecule on the surface of the T cell (Figure 12.2a/b).

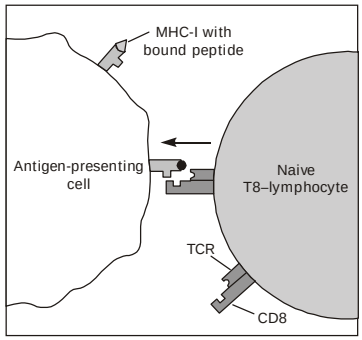


Fig. 12.2a. The MHC class I–antigen-T8 cell receptor interaction.

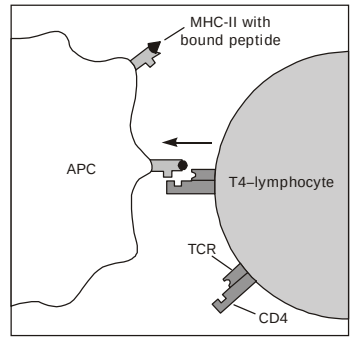


Fig. 12.2b. The MHC class II–antigen-T4 cell receptor interaction.

This interaction then generates a series of biochemical reactions which are aimed at accomplishing two important events : (*i*) the secretion of interleukin–2 (IL–2), by the T cell and (*ii*) the expression of receptors for IL–2 on the T cell surface. The initial interaction between T cell receptor and antigen stimulates membrane bound phospholipase C to hydrolyze membrane bound inositol (Figure 12.3). This results in the generation of two biologically active metabolites: inositol triphosphate (IP3) and diacylglycerol. The inositol triphosphate increases cytoplasmic free calcium and the diacylglycerol activates the enzyme protein kinase C. These two metabolites, are responsible for activating T cells. An activated T cell is then able to transcribe, translate and express two important gene products: the IL–2 molecule and the IL–2 receptor. Thus IL–2 is secreted and the IL–2 receptor is expressed on the cell surface. When IL–2 and its receptor interact on the cell membrane, T cell proliferation occurs (Figure 12.4). Activated T cells secrete several other lymphokines (discussed in Chapter 13), including interferon γ, which help regulate the T cell cycle by the interleukin - interferon circuit.

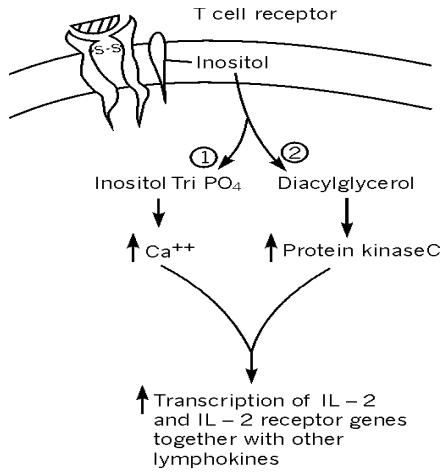


Figure 12.3. Biochemical events in *T* cell activation

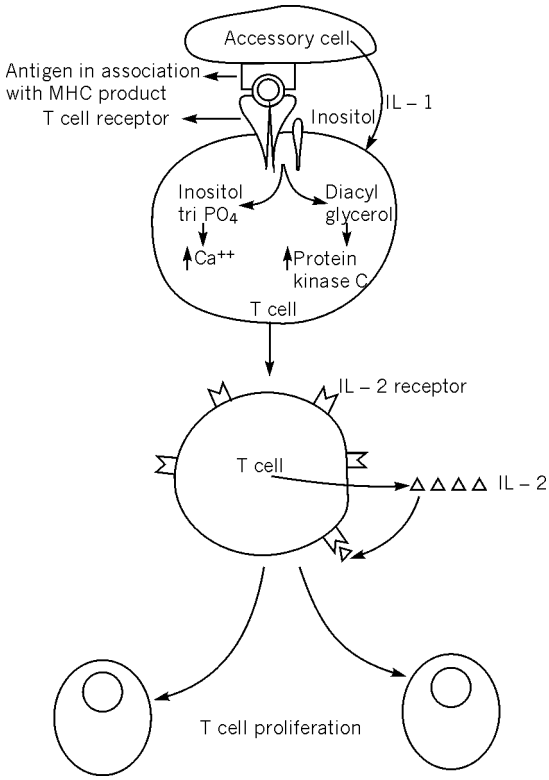


Figure 12.4. T cell proliferation.

Co-stimulatory signals

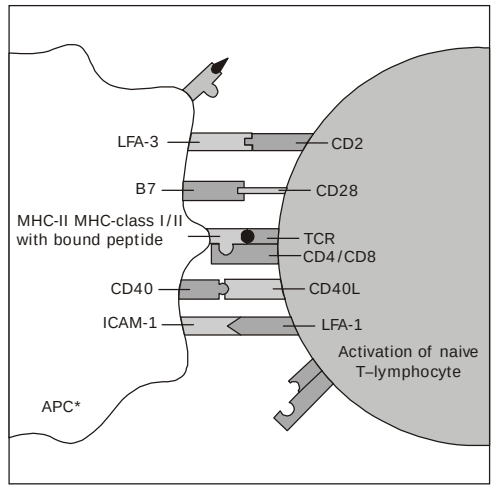
In addition, certain cell adhesion molecules have been identified that are necessary for efficient interaction between T lymphocytes and accessory cells or target cells. These co-stimulatory signals involve the interaction of accessory molecules on the APC with their corresponding ligands on the T lymphocytes (Figure 12.5) These co-stimulatory molecules are only synthesized when toll-like receptors on APCs bind to pathogen-associated molecular patterns of microbes (Refer Chapter2: Innate immunity). Cell adhesion molecules and their ligands are presented in Table 12.1.

Table 12.1: Cell adhesion molecules (on the APC) and their ligands on T cells

Dendritic cell or macrophage	*T lymphocytes*
MHC I-peptide	TCR/CD8
MHC II - peptide	TCR/CD4
ICAM-1	LFA-1
B 7-1 and B7-2	CD28
LFA-3	CD2

The importance of lymphocyte function associated (LFA – 1) molecule is evidenced by experiments with monoclonal antibodies which block LFA - 1. This leads to inhibition of cytotoxic T cell activity, natural killer cell (NK) activity and antibody dependant cellular cytotoxicity (ADCC). Patients deficient in this factor suffer life threatening bacterial and fungal infections. The protein which is believed to be the ligand for LFA-1 is the inter cellular adhesion molecule–1 (ICAM–1). Antibodies that block ICAM–1 produce the same effects as blockage of LFA–1. Various cytokines like IL–1 and IFN γ increase ICAM expression on the B cell surface. The CD2 ligand is the LFA–3 molecule. Blockage of these molecules also decreases T cell killing activity by blocking T cell–target cell adhesion. The CD4 and CD8 ligand associations with their corresponding MHC molecules have already been described in previous sections.

Molecular interactions between the APC and the T8 - lymphocyte produce signals and cytokines for activation of the naive T8–lymphocyte. Once activated, signals and cytokines from effector T4–lymphocytes, primarily T_h1 cells, will then be able to activate the T8–lymphocyte; turn on genes responsible for the proliferation and differentiation of that T8-lymphocyte into an effector cell: the functioning cytotoxic T–lymphocyte (CTL).



* APCs can also be B cells bearing antigen bound to surface Ig (sIg)

Figure 12.5 Co-stimulatory molecules that enhance Tcell–APC interaction.

Interleukin - Interferon circuit

The interleukin–interferon circuit is graphically depicted in Figure 12.6. Antigens are processed and presented to T cells by antigen presenting cells such as macrophages. This shifts the resting T lymphocytes from the G0 state to the early G1 phase of the cycle. G1 cells synthesize receptor inducing factors and other proteins. Under the influence of IL-1 from the accessory cells, some of the early G1 cells proceed into the late G1 phase of the cell cycle, where IL–2 promotes expression of IL–2 receptors on late G1 cells. Other lymphocytes moving in a similar cell cycle are stimulated by IL–1 to secrete IL–2. IL–2 in turn stimulates IL–2 receptor bearing cells to proliferate or actively replicate and enter what is known as the GS (synthesis) phase in T cell activation. Actively replicating T lymphocytes secrete interferon γ (IFN γ). IFN γ has a positive feed back effect on IL–1 production, antigen processing and expression of MHC Class II molecules, thus promoting accessory cell functions resulting in a cyclical increase of activated T cells predominantly of the T_h1 subset.

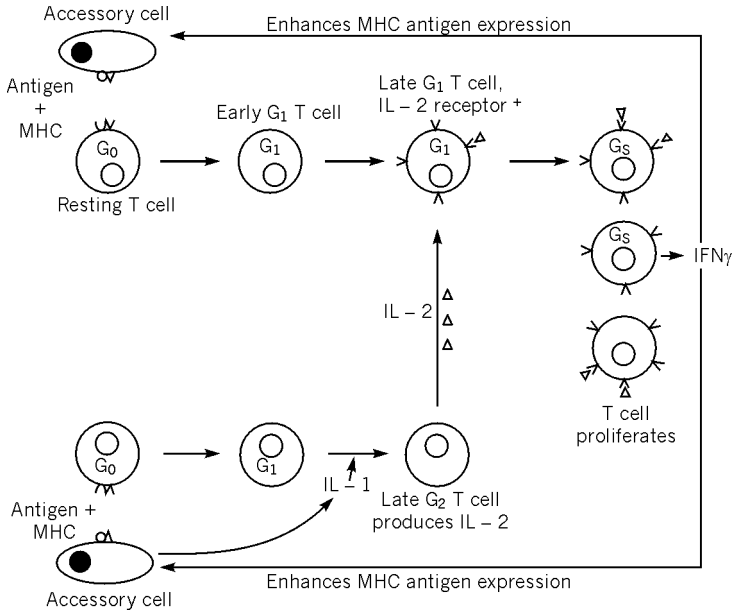


Figure 12.6. The Inter leukin–Interferon circuit.

Activation of B Cells

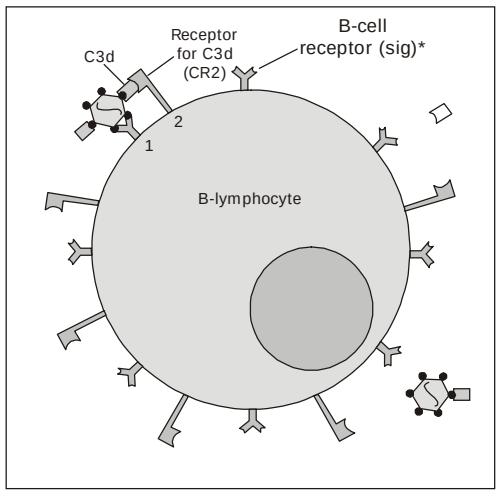
It is now evident, that for many B cells to make antibody to a particular antigen, T cell help is essential. Such antigens are also called **T dependant** antigens. There are others, notably the capsule of the organism *Streptococcus pneumoniae* which are **T-independent** antigens and can evoke antibody production from B cells without T cell help.

Activation in response to T- dependant antigens

When T cell help is to be rendered, it has been shown that T cells have first to be activated by a separate process which does not involve B cells, as we have just seen in the previous section. Pioneering experiments by A. Mitchison in 1971, showed that, for helper T cells and B

cells to collaborate, they must, each in turn, recognize parts of the same antigen molecule. This, and other experiments have provided compelling evidence that the activated helper T cell needs to be physically close to the B cell.

The first signal for the activation of a naive B-lymphocyte occurs when B-cell receptors, the surface immunolobulin (slg), on the surface of the B-lymphocyte bind epitopes of T-dependant antigens having a corresponding shape. A second signal is also needed for the activation of the naive B-lymphocyte. This is provided when a component of the complement system , C3b binds to the microbial surface. C3b is subsequently degraded to C3d which, in turn, binds to a complement receptor called CR2 on the surface of the B-lymphocyte (Figure 12.7a). Activation of the naive B cell triggers a series of metabolic events in the B cell, similar to what has been discussed for the T cell; the result is gene activation and increased production of MHC Class II molecules, co-stimulatory substances such as B7 and CD40 and receptors for IL-2 and interleukins 4 to 6 (IL-4, IL-5, IL-6); required for B cell growth and differentiation.



* slg = Surface Immunoglobulin

Figure 12.7a. Activation of the naive B lymphocyte.

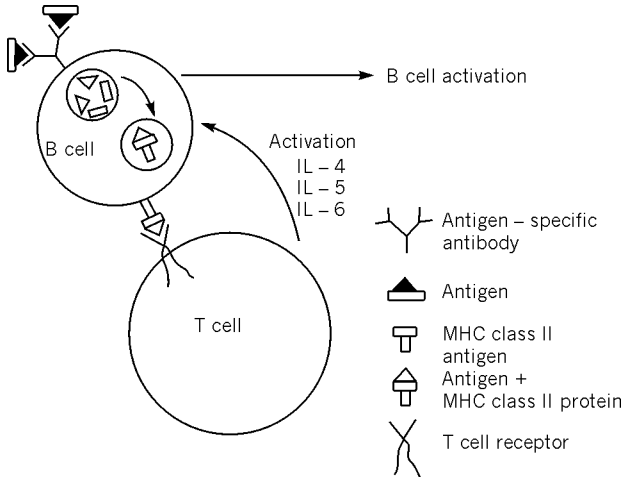


Figure 12.7b. T cell-B cell interaction.

Antigens bound to surface immunoglobulins on the B cell are internalized and degraded to oligopeptides which bind to MHC Class II proteins within the cell. The antigen peptide–MHC Class II complex then goes to the cell surface, where it is recognized by an antigen specific T cell receptor on the activated helper T cell, primarily the T_h2 cell (Figure 12.7b). The B cell, having displayed antigen in conjunction with the MHC Class II molecule, has now moved from the B0 stage to the B1 stage in its cycle (Figure 12.8). Here co-stimulatory molecules such as CD28 and CD40L on the T_h2 cell bind to B7 and CD40 molecules on the activated B lymphocyte (Figure 12.5). As a result of this activated T helper cell - B cell interaction, the T_h2 cells produce cytokines such as IL-4, IL-5, IL-6. Collectively these cytokines enable activated B-lymphocytes to proliferate stimulate activated B-lymphocytes to synthesize and secrete antibodies and promote the differentiation of B-lymphocytes into antibody-secreting plasma cells. T_h2 cells also enable B-lymphocytes to undergo affinity maturation through somatic hypermutation and to switch antibody classes, that is, produce either IgG, IgA, or IgE. This allows the B-lymphocytes to "fine-tune" the shape of the antibody for better fit with the original epitope and to produce the class of antibody of greatest value for that site.

B cells move into the B2 stage of activation and display receptors for IL-4 and IL-5. When the receptors and the corresponding interleukin molecules interact, B cells grow and proliferate yielding several clones of B3 cells. B3 cells in turn display receptors for IL-6 and other related cytokines. Interaction of these cytokines with their receptors on the B cell pushes the B cell into the final differentiation stage– the plasma cell, and antibody production ensues. A set of B3 cells however, remain unstimulated by IL-6 and other related cytokines, albeit with the receptors displayed. This pool of B3 cells constitutes the **memory cells**. Subsequent exposure of B cells to antigen will yield a much faster and greater antibody response since the terminal differentiation stages before antibody production are all set. To go into production, these B cells only need renewed fuelling with IL-6 and related cytokines.

Other agents which induce polyclonal B cell proliferation are Epstein - Barr virus, *Chlamydia trachomatis* and lipopolysaccharides which react with non immunoglobulin receptors on the B0 cell.

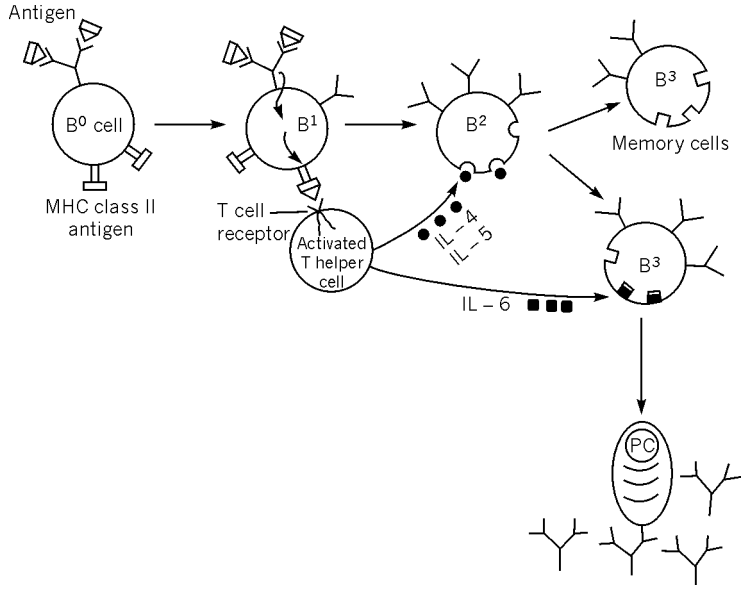


Figure 12.8. Events involved in B-cell activation.

Activation in response to T- independent antigens

T-independent antigens are usually large carbohydrate and lipid molecules with multiple, repeating subunits. B-lymphocytes mount an antibody response to T-independent antigens without the requirement of interaction with T4-lymphocytes. Bacterial LPS, from the gram-negative cell wall, and capsular polysaccharides (as in *S. pneumoniae*) are examples of T-independent antigens. The resulting antibody molecules are generally of the IgM isotype and do not give rise to a memory response. There are two basic types of T-independent antigens: TI-1 and TI-2.

TI-1 antigens include lipopolysaccharide (LPS) from the outer membrane of the gram-negative cell wall and bacterial nucleic acid. These antigens activate B-lymphocytes by binding to their specific toll-like receptors (see chapter 2) rather than to B-cell surface immunoglobulin receptors. Antibody molecules generated against TI-1 antigens are often called “natural antibodies” because they are always being made against bacteria present in the body.

TI-2 antigens, such as capsular polysaccharides, are molecules with multiple, repeating subunits. These repeating subunits activate B-lymphocytes by simultaneously cross-linking a number of B-cell receptors.

CHAPTER – 13

CYTOKINES

The course of immunologic and inflammatory pathways is mediated by several hormone like soluble substances that are secreted by the concerned cell types. These substances have been given a general term: the **cytokine**. Those secreted by lymphocytes are called **lymphokines**, those produced by monocytes or macrophages are called **monokines**.

Cytokines are low molecular weight, soluble proteins that are produced in response to an antigen and function as chemical messengers for regulating the innate and adaptive immune systems. They are produced by virtually all cells involved in innate and adaptive immunity, but especially by T helper lymphocytes. The activation of cytokine-producing cells triggers them to synthesize and secrete their cytokines. The cytokines, in turn, are then able to bind to specific cytokine receptors on other cells of the immune system and influence their activity: this includes both enhancing and suppressing responses.

Cytokines maybe characterized as **pleiotropic**, **redundant** or **multifunctional**.

- Pleiotropic cytokines can act on a number of different types of cells rather than a single cell type.
- Redundant defines the ability of a number of different cytokines to carry out the same function.
- Multifunctional cytokines are able to regulate a number of different functions.

Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can act synergistically or antagonistically.

There are three functional categories of cytokines depending on whether they

(a) Regulate innate immune responses
(b) Influence adaptive immune responses
(c) Stimulate haematopoiesis

The description of cytokines below is not intended to be comprehensive nor complete; it provides a snapshot of some of the more commonly known factors and their principal activities. More new cytokines and their receptors are continuously being discovered. A brief description of the important interleukins and interferons is given in Table 13.1

A. Cytokines that Regulate Innate Immune Responses

Cytokines that regulate innate immunity (see Chapter 2) are produced mainly by macrophages and dendritic cells although they can also be produced by T-lymphocytes, NK cells, and other cells. They are produced primarily in response to pathogen-associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS), peptidoglycan monomers, teichoic acids, and double-stranded DNA. Most act on leukocytes and the endothelial cells that form blood vessels in order to promote and control early inflammatory responses.

Tumor necrosis factor (TNF)

TNF-alpha is a key cytokine in the mediation of acute inflammation. In excessive amounts it is the principal cause of systemic complications such as the shock cascade. Functions include acting on endothelial cells to stimulate inflammation and the coagulation pathway; stimulating endothelial cells to produce selectins and ligands for leukocyte integrins during diapedesis; stimulating endothelial cells and macrophages to produce chemokines that contribute to diapedesis, chemotaxis and the recruitment of leukocytes; stimulating macrophages to secrete interleukin-1 (IL-1); activating neutrophils and promoting extracellular killing by neutrophils; stimulating the liver to produce acute phase proteins; and acting on muscles and fat to stimulate catabolism for energy conversion. In addition, TNF is cytotoxic for some tumour cells; it interacts with the hypothalamus to induce fever and sleep; stimulates the synthesis of collagen and collagenase for scar tissue formation; and activates macrophages. TNF is produced mainly by monocytes, macrophages, dendritic cells and T_h1 cells.

Interleukin-1

The major cell sources of IL-1 are monocytes and macrophages, though several other cell types produce interleukin - like substances. Resting macrophages and circulating monocytes produce little IL-1. When macrophages are stimulated, IL-1 activity increases markedly. Stimulants of IL-1 are particles like silica and adjuvants such as LPS or muramyl dipeptide (MDP). All the agents that activate T-lymphocytes directly, such as antigen in conjunction with MHC molecules also stimulate IL-1 release. T and B cell products such as interferon γ, colony stimulating factor and antigen - antibody complexes are potent stimulators of IL-1.

Interleukin-1 release is blocked by drugs such as hydrocortisone and cyclosporin, probably accounting for the anti inflammatory and immunosuppressive properties of these drugs. Excessive doses of the macrophage stimulators can have an inhibitory effect on IL-1. Prostaglandin E2 has been shown to have a negative effect on IL-1 production.

Biological properties of IL-1

(*i*) *On T cell function*

IL-1 augments the proliferation of medullary thymocytes and increases the production of lymphokines in peripheral T cells. It increases T lymphocyte production of colony stimulating factor and other chemotactic factors. Hence less differentiated T cells proliferate and more differentiated T cells display increased secretory activity. IL-1 increases the accessibility of the antigen binding receptor and augments antigen stimulated CD4+ and CD8+ T cells to produce IL-2. It therefore increases the tumoricidal and bactericidal functions of T cells.

IL-1 in conjunction with IL-2 or interferon, enhances non specific **natural killer cell** activity of large granular lymphocytes which in turn have tumoricidal activity.

(*ii*) *On B cell function*

IL-1 augments proliferation, differentiation and antibody producing functions of B lymphocytes. It also induces expression of surface bound immunoglobulin receptors. IL-1 promotes B cell antibody production directly and by augmenting T cell helper function.

(*iii*) *On non-lymphocytic cells*

IL-1 promotes the growth and function of non lymphoid cells as well. IL-1 can act as an endogenous pyrogen, probably by stimulating prostaglandin production. This could explain the

muscle wasting and cachexia of chronic febrile illness. IL-1 stimulates hepatocytes to produce acute phase proteins such as fibrinogen,C- reactive protein, caeruloplasmin and α1 antitrypsin.

On inflammatory cells, IL-1 causes neutrophilia, is chemotactic and induces release of lysozyme and lactoferrin. It stimulates the production of superoxide anion via the hexose monophosphate (HMP) stunt. IL-1 increases bone resorption and collagen synthesis and is important in fibrosis and wound healing.

Certain non macrophage cells produce IL-1 like substances. They are keratinocytes, epithelial cells from the oral mucosa and cornea, astrocytes from the brain and mesangial cells from the kidney.

Chemokines

Chemokines are a group of cytokines that enable the migration of leukocytes from the blood to the tissues at the site of inflammation. They increase the affinity of integrins on leukocytes for ligands on the vascular wall during diapedesis, regulate the polymerization and depolymerization of actin in leukocytes for movement and migration, and function as chemoattractants for leukocytes. In addition, they trigger some white blood cells (WBCs) to release their killing enzymes for extracellular killing and induce some WBCs to ingest the remains of damaged tissue. Chemokines also regulate the movement of B-lymphocytes, T-lymphocytes, and dendritic cells through the lymph nodes and the spleen. Certain chemokines have also been shown to suppress HIV, probably by binding to chemokine receptors (e.g. CCR5) serving as the second binding factor for HIV on $CD4^+$ cells. When produced in excess amounts, chemokines can damage healthy tissue as seen in rheumatoid arthritis, pneumonia, asthma, adult respiratory distress syndrome (ARDS), and septic shock. Examples of chemokines include IL-8, macrophage activating factors: macrophage inflammatory protein (MIP-1a), MIP-1b; monocyte chemoattractant protein (MCP-1), MCP-2, MCP-3, growth related oncogene (GRO-a), GRO-b, GRO-g, and the molecule 'regulated on activation-normal T cell expressed and secreted' (RANTES). Chemokines are produced by many cells including leukocytes, endothelial cells, epithelial cells and fibroblasts.

Interleukin-12 (IL-12)

IL-12 is a primary mediator of early innate immune responses to intracellular microbes. It induces cell-mediated immunity. It functions as a stimulant for the synthesis of interferon-gamma by T-lymphocytes and NK cells; increases the killing activity of CTLs and NK cells; and stimulates the differentiation of naive T4-lymphocytes into interferon-gamma producing T_h1 cells. It is produced mainly by macrophages and dendritic cells.

Type I Interferons

Originally discovered in 1957 by Isaacs and Lindenmann, interferons were found to consist of a family of proteins which share a unique antiviral property. These same proteins also exert non viral functions such as those of an antiproliferative and immunoregulatory agent.

Types of Interferons

Interferons can be classified according to their primary cell or origin

- interferon α from leukocytes,
- interferon β from fibroblasts,
- interferon γ from T lymphocytes.

They can also be classified as type I and type II according to the agents that stimulate their release. Type I interferons (IFN α and IFN β) are induced by viral infection or artificially by double stranded RNA (ds RNA). Lymphoblastoid cells produce IFN α and β. Both α and β interferons are acid stable. Type II interferon (discussed later), also known as IFN γ or immune interferon, is stimulated by foreign antigen or mitogen. The genes coding for the three interferons have been cloned and sequenced.

Interferon α is produced when leukocytes and lymphocytes are exposed to viruses. It consists of multiple sub species that are antigenically related. By recombinant DNA technology it has been shown that more than 20 distinct interferon α genes and polypeptides exist.

Interferon β is antigenically distinct and is the predominant species elaborated by fibroblast cells. Epithelial cells and macrophages also secrete interferon β. There are several types of IFN β species.

Mechanisms of action of the interferons

Interferons act by binding to specific receptors on the cell surface. Subsequent to binding, the interferon - receptor complex is internalized by receptor mediated endocytosis. Type I interferons, produced by virtually any virus-infected cell, provides an early innate immune response against viruses. Interferons induce uninfected cells to produce enzymes capable of degrading mRNA. These enzymes remain inactive until the uninfected cell becomes infected with a virus. At this point, the enzymes are activated and begin to degrade both viral and cellular mRNA. This not only blocks viral protein synthesis, it also eventually kills the infected cell. They also promote body defences by enhancing CTL, macrophage, dendritic cell, NK cell and antibody-producing cell activity.

Type I interferons also induce MHC-I antigen expression needed for recognition of antigens by CTLs; augment macrophage, NK cell, CTL, and B-lymphocyte activity; and induce fever. Interferon-alpha is produced by T-lymphocytes, B-lymphocytes, NK cells, monocytes/ macrophages; interferon-beta by virus-infected cells, fibroblasts, macrophages, epithelial cells and endothelial cells.

Immunoregulatory effects of Type I interferons

The immunoregulatory effects of interferons in general are exerted on cells that carry out the hosts' defence strategy - the macrophages, T and B cells and large granular lymphocytes with natural killer cell activity, NK cells.

(a) On macrophages

Interferons increase the bactericidal and tumoricidal capabilities of macrophages and augment their accessory function of antigen presentation. The activation of macrophages by IFN α, β or γ is accompanied by increased expression of receptors for the Fc portion of the immunoglobulins (FcR). This increased expression of FcR promotes both increased phagocytosis of immune complexes and the increased capacity of macrophages to lyse antibody coated bacteria, viruses, parasites and tumour cells. This phenomenon is known as antibody dependant cellular cytotoxicity (ADCC).

All three interferons have been reported to either increase or decrease synthesis and secretion of multiple proteolytic enzymes by macrophages, depending on the type of stimulus. This endows the host with the capacity to destroy or detoxify invading agents. By increasing the intracellular proteolytic enzymes, antigen processing also gets enhanced, as this requires proteolytic cleavage of whole antigens.

(b) On large granular lymphocytes and natural killer activity

Natural killer (NK) activity is a term used to describe cytotoxic activity of large granular lymphocytes without prior sensitization with specific antigen. NK activity is primarily directed against virus infected cells, certain tumour cell lines and normal effete haemopoietic cells. All three interferons enhance NK cell activity in large granular lymphocytes. There are several theories regarding the mechanisms by which these cells become more cytocidal under the influence of interferon. These include: increased expression of recognition structures on target cells, increased binding between these lymphocytes and target cells, increased metabolic activity and the production of cytolytic granules by large granular lymphocytes.

Animal studies have demonstrated that interferon exerts in vivo anti tumour activity. Anti tumour activities of interferon have been attributed both to anti proliferative effects as well as to augmentation of host anti tumour responses.

Interferon assays

Human interferon assays can be used for diagnostic purposes, as in the diagnosis of viral infections and tuberculosis using the ELISA or cytokine assays such as the ELISPOT (see Chapter 8). Serum interferon levels rise in a variety of viral infections and after immunization; they may be increased during high fever. Interferon has been detected in CSF in cases of viral meningitis. Serum of patients with autoimmune disease show raised levels of IFN γ. Studies have shown that neonates have the ability to produce α and β interferons; production of γ interferon is usually deficient. Patients receiving powerful immuno suppressives, as in transplant patients, have decreased quantities of α and γ interferons. Patients with malignant lesions, especially the lymphocytic leukaemias and those on chemotherapeutic drugs have a decreased ability to produce α and γinterferon. Diseases like uraemia, systemic lupus erythematosus and multiple sclerosis have an adverse effect on interferon production.

Therapeutic uses of interferon

(a) Interferon alpha

Interferon α was the first of the interferons to be used successfully in clinical therapy. There are two types of recombinant IFN-α available (2a and 2b); a newer formulation is called Pegylated Interferon. This drug was developed to counter the rapid breakdown of the original IFN α products. It has five approved indications in the U.S. — hairy cell leukemia, AIDS-related Kaposi's sarcoma, genital warts, chronic hepatitis B and hepatitis C.

Alpha interferon's biggest impact came in 1991 and 1992, when it was licensed for chronic hepatitis C and then for chronic hepatitis B. Alpha interferon works differently in each disease. In hepatitis C the virus invades and destroys liver cells; interferon lowers the virus population to a level where it no longer causes injury. In hepatitis B, however, it is the immune system's attack on virus-infected cells that causes the illness. Interferon helps by stimulating immune cells that in turn repel the invasion. Not all patients respond to interferon therapy; some of them relapse when the drug is stopped.

Although a newer drug has now taken its place, alpha interferon was for a time the main treatment for hairy cell leukemia, a relatively rare blood cancer that usually strikes older men. It is also used to shrink tumors and prolong survival for some AIDS patients disfigured by the purple lesions of Kaposi's sarcoma, a cancer that primarily affects the skin.

Alpha interferon's antiviral action has been impressive against the human papilloma virus in condylomata acuminata (genital warts), the most common sexually transmitted viral disease. In women, the virus is thought to be a precursor for cervical cancer.

(*b*) *Interferon beta*

Ambulatory patients with relapsing-remitting multiple sclerosis who took high doses of beta interferon had about 30% fewer attacks, and half as many severe ones, as people who took a placebo. As a result, treated patients were hospitalized less often. Magnetic resonance imaging showed a marked decrease in the number of new brain lesions in these patients. Side effects of treatment were minimal, with about 75% of patients on a high dose reporting mild flu-like symptoms that diminished over time.

(*c*) *Interferon gamma*

Finally, gamma interferon is used to treat chronic granulomatous disease, a rare, inherited immune disorder found mostly in young men. It is also used to treat malignant osteoporosis. Medical researchers are actively testing interferons — primarily alpha — in more than 150 clinical trials involving patients with various cancers or HIV infection. Preliminary indications are that alpha interferon may benefit patients with early stage melanoma, renal cell carcinoma, chronic myeloid leukemia, and multiple myeloma. The drug also appears to be a team player when combined with other agents; some skin cancers, respond especially well to a combination of alpha interferon and retinoids, which are used now to treat acne.

Side effects of interferon therapy

The most common side effect is that of flu-like symptoms with the first few injections. A more serious side effect is depression; particularly if there is a history of depression. Typical symptoms of depression are unexplained sadness, spells of crying, insomnia, early morning awakening, loss of interest in food, sex, hobbies, etc. Interferon may affect the bone marrow and can be severe. Rarely, the thyroid gland may become over or underactive. Extreme fatigue or insomnia, irritability, or excessive sweating may be the warning signs. Other troublesome side effects may include nausea, diarrhoea, thinning of hair, irritation at injection site and weight loss. For the most part these are reversible with time and occasional withholding of the medication.

Interleukin-6 (IL-6)

Interleukin-6 is a multifunctional lymphokine that regulates immune responses, the acute phase reaction and haematopoiesis. It stimulates the liver to produce acute phase proteins; and increases neutrophil production. IL-6 aids B cell proliferation and induces the terminal maturation of B cells into immunoglobulin - secreting cells. In addition, IL-6 activates T cells and induces their proliferation. In association with IL-2 and IFN γ, IL-6 induces differentiation of cytotoxic T cells. IL-6 is produced by many cells including T-lymphocytes, macrophages, monocytes, endothelial cells and fibroblasts.

Interleukin-10 (IL-10)

IL-10 is an inhibitor of activated macrophages and dendritic cells and as such, regulates innate immunity and cell-mediated immunity. IL-10 inhibits their production of IL-12, co-stimulator molecules, and MHC-II molecules, all of which are needed for cell-mediated immunity. IL-10 is produced mainly by macrophages, and T_h2 cells.The formation of granuloma in infections such as tuberculosis, leprosy, histoplasmosis, and coccidioidomycosis is a cytokine-mediated

cellular response. Because macrophages have difficulty in removing the microbes that cause these infections, there is a continuous secretion of cytokines and chemokines that leads to an accumulation of densely packed macrophages around the microbes. The macrophages release fibrogenic cytokines such as TNF and IL-1 that lead to the formation of granulation tissue and scar tissue. The resulting mass is called a granuloma and is an attempt by the body to "wall-off" or localize the infection.

B. Cytokines that Regulate Adaptive Immune Responses (Humoral and Cell-Mediated)

Cytokines that regulate adaptive immunity are produced primarily by T-lymphocytes that have recognized an antigen specific for that cell. These cytokines function in the proliferation and differentiation of B-lymphocytes and T-lymphocytes after antigen recognition.

Interleukin-2 (IL-2)

The cellular origin of IL-2 is the mature T lymphocyte. T helper and T cytotoxic subsets of T cells can both produce IL-2. Large granular lymphocytes also secrete IL-2. Three signals are necessary for IL-2 release : MHC restriction, interaction of antigen and the T cell receptor and IL-1 (Figure 12.6). The pathway to T cell activation and the pivotal role played by IL-2 has been discussed in the previous chapter.

Several substances that have be shown to be suppressive, seem to exert their effects primarily by inhibiting IL-2 gene expression. Included in this category are glucocorticoids, cyclosporine and prostaglandin E2. Impaired production of IL-2 has been reported in several patients with deficiencies in cellular immunity; as in leprosy, AIDS and in patients with metastatic cancers.

Resting lymphocytes do not bind to IL-2. The development of a response to IL-2 requires denovo acquisition of membrane receptors for IL-2. Upon appropriate activation, T lymphocytes develop IL-2 receptors in 6 hours. For T cell proliferation to occur, IL-2 needs to couple with its receptor. The secretion of IL-2 by an activated T cell is strictly regulated by external stimuli (antigen provocation).

Functions of IL-2 on T cell clones

The addition of IL-2 to activated T cells promotes proliferation.The IL-2 dependant T cells–predominantly T helper cells -produce other lymphokines such interferon, colony stimulating factor and interleukin -3; they produce factors for B cell growth and differentiation. IL-2 activated T cells also exhibit enhanced cytotoxicity showing that besides clonal expansion, IL-2 also promotes cellular function such as delayed hypersensitivity and T cell cytotoxicity.

IL-2 action on non T lymphocytes

Although they lack T cell markers, large granular lymphocytes that exhibit natural killer cell activity (see Chapter:14), also respond to IL-2. These lymphocytes begin to grow, produce lymphokines and exhibit enhanced natural killer activity. Receptors for IL-2 have also been found on B cells. IL-2 can increase antibody production as well as proliferation in these cells. Finally macrophages can also be induced to become cytocidal with large doses of IL-2.

Interleukin-4 (IL-4)

Interleukin-4 is a 20kd glycoprotein that affects many cell types including B cells. It pushes B cells out of the resting phase into activation. It induces B cells to express MHC Class II

antigens; CD23 which is a low affinity receptor for IgE; and IL-4 receptors. Antibody synthesis is also influenced by IL-4. IL-4 inhibits the switch from IgM to IgG. IL-4 is a major stimulus for production of IgE and the development of T_h2 cells for defence against helminths and arthropods. It also antagoizes the effects of interferon-gamma and thus inhibits cell-mediated immunity. IL-4 is produced mainly by T_h2 cells and mast cells.

Interleukin-5 (IL-5)

IL-5 is a growth and activating factor for eosinophils as a defence against helminths and arthropods. It also stimulates the proliferation and differentiation of antigen-activated B-lymphocytes and the production of IgA. IL-5 is produced mainly by T_h2 cells.

Interferon gamma

Type II interferon, i.e. IFN γ, is produced primarily by T_h1 cells, $CD8^+$ cells, and NK cells as part of the immune response and functions mainly to promote the activity of the components of the cell-mediated immune system. Interferon γ is an acid labile molecule. There is only one type of IFN γ.

IFN-gamma is the principal cytokine for activating macrophages. It also induces the production of MHC-I molecules, MHC-II molecules, and co-stimulatory molecules by APCs in order to promote cell-mediated immunity (activation of T lymphocytes requires that the T cells recognize antigen in conjunction with Class I and II MHC molecules); it also activates and increases the antimicrobial and tumoricidal activity of monocytes, macrophages, neutrophils, and NK cells. IFN γ more than the other two interferons, increases and induces IL-1 release from monocytes, thus augmenting and amplifying immunologic responses. IFN-γ stimulates the differentiation of T4-lymphocytes intoT_h1 cells and inhibits the proliferation of T_h2 cells; stimulates the production of IgG subclasses that activate the complement pathway and promote opsonization; augments or inhibits other cytokine activities; and exerts weak antiviral activity.

For interferon assays and therapeutic uses of interferons, including IFN – γ, refer discussion on Type I interferons above.

Transforming growth factor-beta (TGF-beta)

TGF-β has a proliferative effect on many mesenchymal and epithelial cell types. Under certain conditions TGF-β will demonstrate anti-proliferative effects on endothelial cells, macrophages, and T- and B-lymphocytes. Such effects include decreasing the secretion of immunoglobulin and suppressing hematopoiesis, myogenesis, adipogenesis and adrenal steroidogenesis. Several members of the TGF-β family are potent inducers of mesodermal differentiation in early embryos.

Interleukin-13 (IL-13)

IL-13 increases the production of IgE by B-lymphocytes, inhibits macrophages, and increases mucus production. IL-13 is made primarily by T_h2 cells.

C. Cytokines that Stimulate Haematopoiesis

Produced by bone marrow stromal cells, these cytokines stimulate the growth and differentiation of immature leukocytes.

Colony-stimulating factors (CSFs)

Colony stimulating factors promote the production of colonies of the different leukocytes in the bone marrow and enhance their activity. Examples include granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and macrophage colony stimulating factor (M-CSF). In addition to their role in promoting production of leukocyte colonies, the CSFs also appear to promote their function. For example, when GM-CSF binds to receptors on neutrophils, eosinophils, and monocytes, it activates these cells and inhibits their apoptosis. GM-CSF increases adhesion of these cells to capillary walls during diapedesis, enhances their phagocytosis and extracellular killing, and increases both superoxide anion generation and antibody-dependent cytotoxicity. The various CSFs are produced by T-lymphocytes, macrophages, and other cells.

Both G-CSF and GM-CSF are available commercially. They are used primarily to decrease neutropenic infections and the resulting morbidity and mortality as a result of chemotherapy and or total body irradiation in bone marrow transplant recipients; in patients with haematological and other malignancies.

Stem cell factor

Stem cell factor makes stem cells in the bone marrow more responsive to the various CSFs. It is made mainly by bone marrow stromal cells.

Interleukin-3 (IL-3)

Interleukin-3 is also called multicolony stimulating factor (multi-CSF). It is produced by T cells and affects growth of macrophages, granulocytes and host cells.

Interleukin-7 (IL-7)

Interleukin-7 affects early B cells. It induces proliferation but not maturation of certain B cell progenitors. It also stimulates the proliferation of early T cells and augments some in vitro T cell responses. Il-7 is produced mainly my fibroblasts and bone marrow stromal cells.

Table 13.1 Interleukins and interferons: primary source and principal activity

Interleukins	Principal Source	Primary Activity
IL1	macrophages and other antigen presenting cells (APCs)	Co-stimulation of APCs and T cells, inflammation and fever, acute phase response, haematopoiesis
IL-2	activated T_h1 cells, NK cells	proliferation of B cells and activated T cells, NK functions
IL-3	activated T cells	growth of haematopoietic progenitor cells
IL-4	T_h2 and mast cells	B cell proliferation, eosinophil and mast cell growth and function, IgE and class II MHC expression on B cells, inhibition of monokine production
IL-5	T_h2 and mast cells	eosinophil growth and function

Interleukins	*Principal Source*	*Primary Activity*
IL-6	activated T_h2 cells, APCs, other somatic cells	acute phase response, B cell proliferation, thrombopoiesis, synergistic with IL-1 and TNF on T cells
IL-7	thymic and marrow stromal cells	T and B lymphopoiesis
IL-8	macrophages, other somatic cells	chemoattractant for neutrophils and T cells
IL-9	T cells	haematopoietic and thymopoietic effects
IL-10	activated T_h2 cells, $CD8^+$ T and B cells, macrophages	inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IL-11	stromal cells	synergisitc haematopoietic and thrombopoietic effects
IL-12	B cells, macrophages	proliferation of NK cells, INF-γ production, promotes cell-mediated immune functions
IL-13	T_h2 cells	IL-4-like activities

Interferons	*Principal Source*	*Primary Activity*
IFN α and β	macrophages, neutrophils and some somatic cells	antiviral effects, induction of class I MHC on all somatic cells, activation of NK cells and macrophages
IFN γ	activated T_h1 and NK cells	induces class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, antiviral effects

CHAPTER – 14

CELL–MEDIATED IMMUNITY

When the potent defence mechanism available in antibody mediated immunity was fully elaborated, scholars in immunology felt that the complete defence strategy of the host against invading micro organisms had finally been worked out. It was soon realized that this form of defence was woefully inadequate when it came to mounting an attack on intra cellular bacteria and viruses, complex structures such as parasites and the body's own cells when they became "effete" or turned malignant. In all of these instances, antigen is not overt but masked within the body's own cell systems. Cell mediated immunity has therefore been targeted to:

- killing intracellular organisms
- destruction of tumour cells
- rejection of graft tissue and
- a delayed type hypersensitivity reaction after contact with certain antigens such as poison ivy and certain topically applied drugs.

Cell mediated immunity involves three major defence strategies:

1. **Activating antigen-specific cytotoxic T-lymphocytes** (CTLs) that are able to lyse body cells displaying epitopes of foreign antigen on their surface. Examples are virus-infected cells, cells with intracellular bacteria and cancer cells displaying tumour antigens. This has been discussed in detail in Chapter: 12. A short summary will be provided here.
2. **Activating macrophage, NK cells and antibody dependant cytotoxicity** enabling destruction of intracellular pathogens.
3. **Stimulating cells to secrete a variety of cytokines** that influence the function of other cells involved in adaptive immune responses and innate immune responses. These have been detailed in Chapter13 and will be summarised here.

1. Activating Antigen-specific Cytotoxic T-lymphocytes (CTLs)

The key stages involved in cell mediated immunity are:

Secretion of lymphokines by T_h1 lymphocytes of the T4 lymphocyte subset

T_h1-lymphocytes recognize antigens presented by macrophages and function primarily to activate and heighten cell-mediated immunity (Figure: 14.1) by producing cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN-gamma) and tumour necrosis factor-beta (TNF-beta). Collectively these cytokines enable T8-lymphocytes to proliferate and differentiate into cytotoxic T-lymphocytes capable of destroying infected host cells and mutant cells; activate cytotoxic T-lymphocytes and NK cells; activate macrophages enabling them to destroy intracellular pathogens; stimulate the production of opsonizing and complement-activating antibodies for enhanced attachment during phagocytosis; activate neutrophils; stimulate

increased production of monocytes in the bone marrow; and function as chemoattractants for phagocytes.

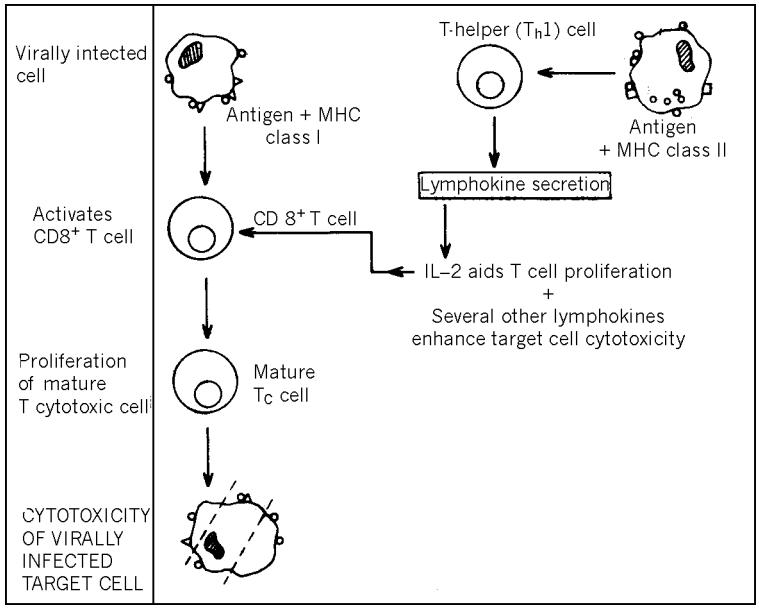


Figure 14.1. The cell mediated immune response.

Cytotoxic T lymphocyte (CTL) activity

Antigen recognition by cytotoxic T cells and their subsequent activation has been greatly discussed in previous chapters. Cytotoxic T cells kill virally infected target cells by direct cell lysis otherwise known as **apoptosis** or programmed cell death. For lysis to occur the target cells must carry both the same viral antigen and the same MHC Class I antigens as the cells that originally induced the proliferation of T cytotoxic cells (Figure 14.2). The T cytotoxic cells recognize antigen in conjunction with MHC Class I molecules, are stimulated by T_h1 helper cells and then turn cytotoxic. The MHC Class I antigens appear to be ideal recognition antigens triggering cytotoxic T cells because they are present on almost all cells. A wide variety of cells can become virally infected or turn malignant, hence it is essential that cytotoxic T cells have a wide repertoire of target cells against which they can mediate their killing action. T cytotoxic cells may thus, be the major effector pathway of cell mediated immunity to certain viruses; this happens just after a few hours of viruses infecting cells—before the viruses can replicate.

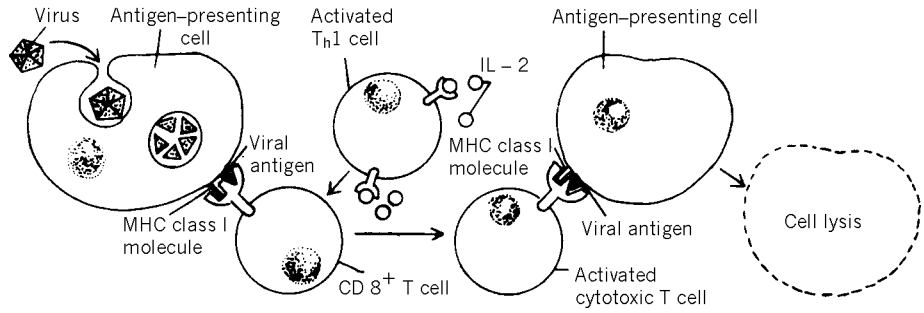


Figure 14.2. T cell - cytotoxicity - a schematic representation.

Mechanism of cell lysis

CTLs and NK cells induce apoptosis by at least 2 different pathways:

The first and most effective pathway involves intracellular granules. The CTLs contain granules composed of proteoglycans to which chemokines are complexed. These granules hold pore-forming proteins called perforins and proteolytic enzymes called granzymes in a protected state. When the TCR and CD8 of the CTL binds to the MHC-I/epitope on the surface of the virus-infected cell, this sends a signal through the CD3 molecule which triggers the release of the perforins, granzymes, and chemokines.

For some years it has been clear that killer cells do their job with great efficiency, first seeking a miscreant target cell, binding to it and then exercising some effect that finally causes cell death, while sparing innocent bystander cells. It is now clear that having bound to its victim, the killer cells shoots it full of holes, more accurately, it fires molecules of a lethal protein at the target cell. It has been shown that a unique pore forming protein is part of the armamentarium of T cytotoxic cells and NK cells.

It is now clear that early in the cell-killing process, granules in the killer cell become concentrated in the part of the cell closest to the target. The granule packaging organelle of the CTL - the Golgi apparatus gets directed towards the contact region and the cell's cytoskeleton is also reoriented towards the target cell. All this happens only when T cell -target cell contact is established and is accompanied by an explosive increase in Ca++ (Figure 14.3). This results in an exocytosis of contents from the killer cell granules now situated at the plasma membrane just apposing the target cell. The killer cell uses exocytosis to fire a lethal agent contained in its granule into the target cell membrane. It has been shown that empty granules are just shell casings for the projectile. This projectile molecule has now been identified as a pore forming protein and is often called "perforin". Once cells are exposed to this 70 kd protein called perforin, in the presence of calcium ions, they lyse within minutes. On the other hand if calcium ions are added to perforin before it makes contact with the target cell, the killing activity is completely abolished.

The perforin molecules secreted by the killer cell insert into the target cell membrane (Figure 14.4). There, within the cell membrane, and in the presence of Ca++ ions, the individual monomeric molecules polymerize into a product that resembles a cylinder or a ring measuring 5-20 nm in diameter. For perforin to damage the target cell, calcium mediated polymerization must take place entirely within the target cell membrane. The reason being, that only the perforin monomer can insert into the target cell. Any secreted perforin that spills into the extra cellular space or blood stream, where calcium is abundant, immediately polymerizes, virtually eliminating "accidental" injury to bystander cells. On the target cell the tubular pores cause leakage of water an ions and finally cell death by lysis. Perforin synthesis has been shown to be stimulated in the presence of interleukin-2.

An interesting parallel has been drawn between perforin and the terminal proteins of the complement cascade. Complement components, C5, C6, C7, C8 and several C9 molecules polymerize to form pores much like those wrought by perforin. Significant homology has been found in the sequence of amino acids between C9 and perforin.

There is now increasing evidence that, though the pore forming protein or perforin is an important method of cell lysis (or it would not have evolved at all!), it is not the central component in lymphocyte mediated killing. A lymphotoxin like activity has been detected in T cells; several other slow killing molecules (granzymes and caspases) have also been identified, secreted by both T cytotoxic cells and NK cells. These molecules are antigenically related to the tumour necrosis factor (TNF).

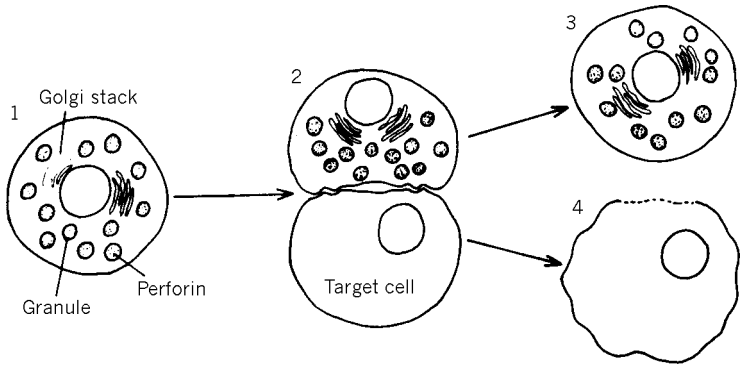


Figure 14.3. The perforin - mediated killing mechanism adopted by the killer lymphocyte. The killer lymphocyte (1) recognizes the target cell and makes close contact with it (2). On contact the cell's granules and the Golgi complex that forms them are reoriented toward the target cell; perforin is secreted and forms pores in the target-cell membrane. Having launched its lethal missiles, the killer cell withdraws and goes on to kill again (3); the damaged target cell dies a programmed death within minutes (4).

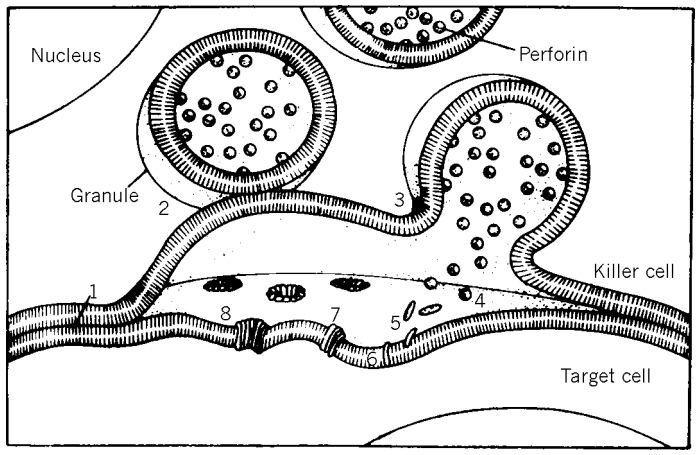


Figure 14.4. Target cell membrane damage by perforin. A rise in the lymphocyte's calcium-ion level, (1), brings about exocytosis, in which the granules fuse with the cell membrane (2) and disgorge their perforin (3) into the small intercellular space abutting the target. Calcium there changes the conformation of the individual perforin molecules, or monomers (4), which then bind to the target-cell membrane (5) and insert into it (6). The monomers polymerize like staves of a barrel (7) to form pores (8) that admit water and salts and kill the cell.

The perforin pores allow granzymes to enter. Certain granzymes, in turn, can then activate the caspase enzymes that lead to apoptosis of the infected cell. The caspases are proteases that destroy the protein structural scaffolding of the cell (the cytoskeleton), degrade the cell's nucleoprotein and activate enzymes that degrade DNA.

Some activated T8-lymphocytes also secrete chemokines (see Chapter 13) that suppress replication of HIV in T4-lymphocytes. (a chemokine receptor is a cofactor for adsorption of HIV to CD4+ cells. When that receptor is occupied by its natural chemokine, HIV cannot adsorb.)

The second pathway by which CTLs can trigger apoptosis of the infected cells is through FasL/Fas interactions. CTLs have a receptor called FasL that can interact with Fas molecules

found on the surface of most cell types. This FasL/Fas interaction triggers an intracellular transduction that activates the caspase enzymes and leads to destruction of the cytoskeleton and chromosome in the infected cell. CTLs are not destroyed in these reactions, they can function over and over again to destroy more virus-infected cells.

Death by apoptosis does not result in release of cellular contents such as inflammatory mediators or viruses as does cell lysis. Instead, the cell breaks into fragments that are subsequently removed by phagocytes. This reduces inflammation and also prevents the release of viruses and their spread into uninfected cells. In addition, the activated enzymes that degrade host DNA can also destroy microbial DNA and thus kill infectious microbes within the cell.

Mechanisms of Evading Cell-Mediated Immunity

A high rate of mutation in some viruses like HIV and the hepatitis C virus (HCV) changes the amino acid sequence and, therefore, the shape of key epitopes. CTLs with TCRs made against the earlier strains of these viruses may no longer bind to and lyse cells infected with mutated strains.

Many viruses such as the cytomegalovirus (CMV) and adenoviruses and some tumour cells can block the formation of MHC-I molecules by the infected cell. As a result, the CTLs are no longer able to recognize that the cell is infected and cannot kill it. Epstein-Barr virus (EBV) down regulates several host proteins involved in attaching viral epitopes to MHC-I molecules and displaying them on the host cell's surface.

Adenoviruses and EBV code for proteins that block apoptosis of the viral infected cell.

2. Activating Macrophages, NK Cells and Antibody Dependant Cytotoxicity

Activated macrophages

Activated macrophages are the effector cells in the cell mediated immune response to various micro organisms. In this effector pathway the macrophage ingests the micro organism, processes the antigen and then presents the antigen on its surface in association with MHC Class II molecules, to T_h1 helper lymphocytes (Figure 14.5). Co-stimulatory molecules such as CD40L on the T_h1 cell then bind to CD40 on the macrophage (Figure 12.5). This triggers the T_h1 cells to secrete several cytokines including interferon-gamma (IFN-gamma). They bind to specific receptors on the macrophage causing its activation. On a subsequent presentation of the same antigen by another macrophage, the sensitized $T_h1$cells are triggered to release several chemokines which attract more macrophages, granulocytes and lymphocytes to the site of the reaction. The ability of activated macrophages to kill micro organisms is enhanced in a non specific fashion. For example a macrophage that has been activated to kill intra cellular *Listeria monocytogenes* would also kill *Salmonella* organisms more efficiently.

Activation of macrophages leads to:

- increased production of toxic oxygen radicals, nitric oxide, and hydrolytic lysosomal enzymes enabling the killing of microbes within their phagolysosomes.
- causes the macrophages to secrete cytokines such as TNF–alpha, IL–1, and IL–12. TNF–alpha and IL–1 promote inflammation to recruit phagocytic leukocytes. IL–12 enables naive T4–lymphocytes to differentiate into T_h1 cells.
- increases the production of B7 co-stimulator molecules (see Chapter 12) and MHC–1 molecules by macrophages for increased T-lymphocyte activation.

- IFN–γ produced by T_h1 cells also increases the production of opsonizing and complement activating IgG to promote enhanced attachment of microbes to phagocytes.

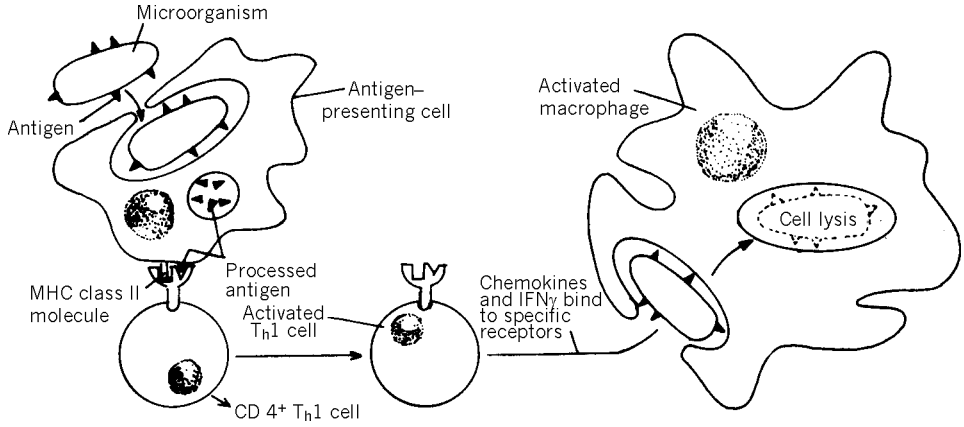


Figure 14.5. Role of activated macrophages in cell mediated immunity.

Natural killer cells

Natural killer cells are large granular lymphocytes that carry the cell surface markers CD56 and CD2. These cells appear to have both anti tumour and anti microbial activity in vivo. NK cells do not require prior exposure to antigen to become cytotoxic and their activity is not MHC restricted. Cytokines such as interleukin–2 (IL–2) and interferon-gamma (IFN-gamma) produced by T_h1 lymphocytes activate NK cells. NK cells also secrete the cytokine IL–1 and in response to IL–2, they become super killers.

NK cells attach to their target cells by a receptor in a calcium independent manner (Figure 14.6). Once the NK cells are activated however, they require calcium for lysis. They cause lysis and apoptosis of the target cell by mechanisms similar to those described for CTLs above. The main target of NK cells are tumour cells and certain virally infected cells.

NK cells appear to use a **dual receptor system** in determining whether to kill or not kill human cells. The first receptor, called the killer-activating receptor can bind to a number of different molecules usually present on nucleated human cells, and this sends a positive signal which enables the NK cell to kill the cell to which it has bound unless the second receptor cancels that signal. This second receptor, called the killer-ihibitory receptor recognizes MHC–I molecules which are also usually present on all nucleated human cells. If MHC–I molecules are expressed on the cell, the killer-inhibitory receptor sends a negative signal that overrides the kill signal and prevents the NK cell from killing that cell.Viruses often suppress class I

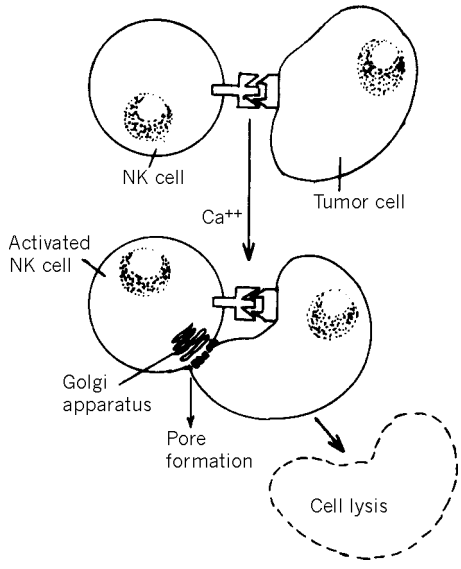


Figure 14.6. Role of NK cells in the cellular immune response.

MHC expression in cells they infect; the virus-infected cell therefore becomes susceptible to killing by NK cells. Tumour cells have reduced or no class I MHC expression, they too, become susceptible to killing by NK cells. The cytomegalovirus (CMV) evades the immune system because it can trigger its host cell to produce altered MHC–I molecules that are unable to bind viral epitopes, and therefore, are not recognized by CTLs. However, NK cells are also unable to kill this infected cell because it is still displaying "MHC-I molecules" on its surface.

Antibody-dependant cell mediated cytotoxicity

NK cells are capable of antibody-dependant cellular cytotoxicity (ADCC). When NK cells are carrying out ADCC, they are sometimes also referred to as killer K cells (K cells). K cells have receptors on their surface for the Fc portion of IgG. The antibody, usually specific IgG against 'foreign' cell antigen, binds to an Fc receptor on the K cell and to an antibody combining site, via the Fab portion, on the target cell (Figure 14.7). The antibody acts like a bridge between the specific antigen bearing target cell and the K cell. The K cell then releases pore-forming perforins, proteolytic enzymes called granzymes and chemokines that cause cell apoptosis and cell lysis similar to the mechanism of killing described for CTLs.

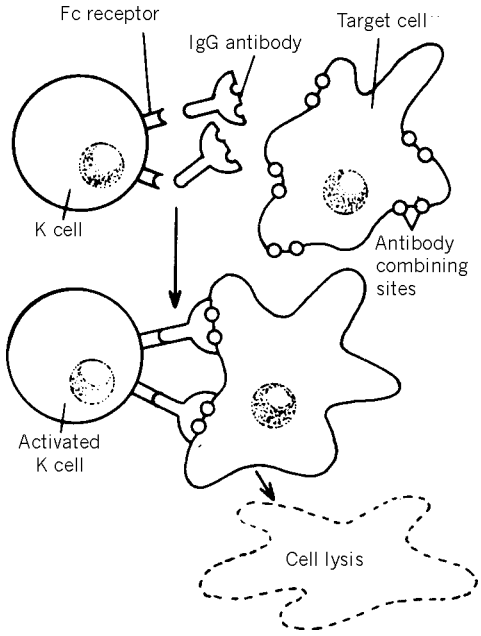


Figure 14.7. Antibody dependant cell mediated cytotoxicity.

Monocytes, neutrophils and eosinophils can also participate in ADCC. Eosinophils, for example, are effective killers of antibody coated schistosomulae, the larval forms of schistosomes. The mechanism of killing involves binding of the eosinophil to the larva via an antibody molecule. The eosinophil releases basic proteins and other molecules from its granules, leading to formation of pores in the target cell membrane. The organism dies because of the leaky membrane. Eosinophils can be activated by both monokines and certain lymphokines.

3. Secretion of cytokines

Cytokines are soluble substances produced by a variety of cell types that amplify and regulate a range of immune responses (Chapter 13). Some cytokines are crucial in cell mediated immune reactions, whereas others play a critical role in antibody responses. A variety of cytokines affect cellular immunity by

- mediating the innate immune response
- regulating haematopoiesis
- directly influencing cell mediated immunity

Cytokines that affect the inflammatory response generally **up-regulate innate immunity**. These cytokines also play a role in mediating resistance to viral infections and causing the cardinal signs of inflammation: pain, redness, swelling, and heat. Included in this group are the Type I Interferons (IFNa and IFNβ), tumor necrosis factor (TNF), interleukin-1, interleukin-6, and interleukin-8.

Type I IFNs include IFN α and IFN β. Type I IFN has several sources and several effects. For simplicity, know that Type I IFN inhibits viral replication in virus-infected cells. IFN α has been used to treat HIV, some forms of cancer, and multiple sclerosis. IFN α is made predominantly by neutrophils, and IFN β is made predominantly by fibroblasts.

Tumor Necrosis Factor (TNF) is made predominantly by monocytes and macrophages following stimulation with bacterial LPS (TNF α) or by activated CD4+ T-cells (TNF β). TNF has many biologic functions including killing tumours and inducing secretion of other inflammatory cytokines. It is probably most important in inducing the production of acute phase proteins by the liver. It also induces fever. This is one of the first cytokines that appear during an inflammatory response.

Interleukin-1 is also made by cells of the monocyte-macrophage cell lineage. It has many of the same functions as TNF, but it typically appears somewhat later in an inflammatory response. Like TNF, IL-1 is an endogenous pyrogen, so it induces fever. IL-1 is also a co-stimulator of CD4+ T-helper cells.

Interleukin-6, like IL-1, has 2 major functions: to mediate inflammation, and to regulate the growth and differentiation of lymphocytes. IL-6 has a function similar to TNF in that it induces the synthesis of acute phase reactants by the liver. In addition, IL-6 also serves as a growth factor for plasma cells.

Interleukin-8, is produced by monocytes. IL-8 is a chemoattractant for neutrophils. It also induces adherence of neutrophils to vascular endothelial cells and aids their migration into tissue spaces.

Interleukin-11 is produced by bone marrow stromal cells and shares the functional activity of IL-6. It is also produced by macrophages and might be anti-inflammatory.

Other chemokines such as macrophage inflammatory protein (MIP-1a), MIP-1b, monocyte chemoattractant protein (MCP-1), MCP-2, MCP-3 enable migration of these cells to the site of inflammation. They also induce certain morphologic, metabolic and functional changes in macrophages, that enhance the cells' ability to kill micro organisms and tumour cells.

Several cytokines play a major role in haematopoiesis in the bone marrow. The best characterized of these are granulocyte/monocyte-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), stem cell factor (SCF), interleukin-3, interleukin-

7 and interleukin-9. Some of these cytokines are being used to treat patients that have deficiencies in haematopoiesis, such as cancer patients who are bone marrow suppressed due to anti-cancer therapy.

GM-CSF and G-CSF are produced by T-helper cells and promote the production of granulocytes (basophils, eosinophils, and neutrophils)(G-CSF), as well as monocytes (GM-CSF).

Interleukin-3 is also produced by T-helper cells and increases the production of basophils and progenitor cells.

Interleukin-7 is produced by bone marrow stromal cells. It induces lymphoid stem cells to differentiate into progenitor B cells. Under some conditions IL-7 stimulates the proliferation of thymocytes.

Stem Cell Factor is also produced by bone marrow stromal cells. It synergizes with a number of hematopoietic growth factors including IL-7, GM-CSF and G-CSF.

Interleukin-9 is produced by T-helper cells. In appears to increase erythropoiesis and mast cell division, and serve as an autocrine growth factor for T-cells.

Specific cellular immune responses are mediated by T-lymphocytes. Activation of T cells requires the presence of antigen. Cytokines serve as 'second signals' to drive the growth and differentiation of antigen-activated lymphocytes. Some cytokines act predominantly on T-cells, others on both T and B cells.

Interleukin-2 is the primary growth and differentiation factor for T-cells. IL-2 causes antigen-primed T-helper cells to proliferate. In addition, it causes antigen-primed cytotoxic T-cells to proliferate and become aggressively cytotoxic. IL-2 is produced by CD4+ T-helper cells.

Type II Interferon or Interferon-γ (IFNγ) is produced by T-helper cells. It is an important cytokine for activating macrophages. An activated macrophage is more phagocytic, it processes and presents antigen more efficiently, it produces more cytokines, and becomes more bactericidal than resting macrophages. Activated macrophages are an important mediator of cellular immunity. IFNγ acts antagonistically against other cytokines such as IL-4.

Interleukin-12 has the opposite effect of IL-10. IL-12 is produced by a number of cells including B-cells, NK-cells, and macrophages. IL-12 enhances IFN production. IL-12 may prove to be a key cytokine in enhancing cell mediated immunity.

For clinical tests used to assess cellular function see Chapter 22.

CHAPTER – 15

HYPERSENSITIVITY

At first glance it would appear as if all immunity: humoral and cellular was geared towards defending the host against unnatural antigens. Nothing but good should come out of a system so precisely planned to weed out aliens and marauders. However, it is painfully evident that reactions to putative foreign antigens may be excessive and that if humoral or cellular immunity is switched on to "high" for any length of time, gross tissue damage can occur. Such reactions have been aptly termed "**hypersensitivity**" reactions. There are two categories of adaptive hypersensitivities: immediate hypersensitivity and delayed hypersensitivity.

Coombs and Gell classified hypersensitivity into four types

Type I : Anaphylaxis

Type II : Antibody dependant cytotoxicity

Type III : Immune - complex mediated disease

Type IV : Delayed type or cell mediated hypersensitivity

Types I, II, III, and IV are reactions which involve the humoral system of antibodies and are sometimes termed "**Immediate hypersensitivity**". Type IV hypersensitivity involves lymphocytes and other cellular components and requires a longer time course to manifest. Because of this, type IV has been termed **delayed type** or **cell mediated hypersensitivity**.

Type I: Anaphylaxis

The most rapid hypersensitivity reaction of the immediate type is known as anaphylaxis. It is characterized by an explosive response occurring within minutes of applying a stimulus and can be generalized or localized. The reactions are mediated by the release of pharmacologically active substances from mediator cells. The primary action of these mediators results in contraction of smooth muscle, increased vascular permeability and increased mucus secretion. These agents cause the **early phase** of allergic reactions that appears within minutes after exposure to the antigen. **Late phase** allergic reactions may begin several hours after exposure to antigen. It is thought that basophils play a major role here. Cell-bound IgE on the surface of basophils of sensitive individuals binds a substance called histamine releasing factor (possibly produced by macrophages and B-lymphocytes) causing further histamine release. That the early and late phase responses are caused by distinct mediators can be shown with inhibitory drugs. Arachadonic acid metabolism inhibitors, such as indomethacin, block only the late response. Sodium cromoglycate which blocks mast cell activation and degranulation blocks both early and late responses.

Generalized anaphylaxis

When a small dose of antigen such as egg white or heterologous animal serum is injected into a guinea pig for the first time, there is no obvious effect. This, however, is called the

sensitizing dose which, in effect, means that the animal has produced IgE antibodies to the antigen and these IgE antibodies are now attached by specific Fc receptors to mast cells and basophils. A second injection of the same antigen given a week or ten days later, results in a dramatic onset of the generalized anaphylactic reaction. The second dose of the antigen has cross linked IgE antibodies situated on mast cells and basophils and caused degranulation. Histamine, together with other mediators released from mast cell or basophil granules causes marked vasodilation and leakage of intra vascular fluids resulting in shock. The manifestations of anaphylaxis vary in different species. The guinea pig will scratch, sneeze, cough, may convulse, go into extreme bronchoconstriction, asphyxiate, collapse and die. On post mortem, the lungs are characteristically over inflated. In the rabbit, the shock organ is the heart and right sided heart failure is the main cause of death. In humans, generalized anaphylaxis presents with itching erythema, vomiting, abdominal cramps, diarrhoea and respiratory distress. In severe cases, laryngeal oedema, vascular collapse and death can occur. Only a timely intravenous injection of adrenaline to counter smooth muscle contraction and capillary dilatation can prevent death.

In allergic individuals, the levels of IgE may be thousands of times higher than in those without allergies. Possibly this is due to a higher number of T_h2 cells which produce IL-4, a cytokine that can increase production of IgE, and a lower number of T_h1 cells that produce gamma-interferon, a cytokine that decreases IgE production.

Local or Cutaneous Anaphylaxis

Upon injection of antigen into the skin of a sensitized animal, a local anaphylactic reaction will occur within a few minutes. It consists of a localized swelling and redness - a wheal and flare reaction. Skin tests in man, for allergy to a wide variety of antigens is an example of this phenomenon. The local increase in vascular permeability, that is characteristic of this reaction may be demonstrated by the use of tracer dyes such as Evans blue which leak out of the vessels at the reaction site and cause “blueing” of the surrounding tissues. These reactions are mediated by histamine and serotonin and are quickly inactivated by plasma histaminases.

Passive Cutaneous Anaphylaxis

Localized anaphylaxis can be passively transferred and forms the basis of the **Prausnitz-Kustner** (P.K.) reaction or test. The P.K. test is based on experiments done by the two scientists after whom the test is named. Kustner was known to be allergic to fish. Serum from Kustner which presumably contained IgE antibodies to fish was injected into Prausnitz who was not allergic to fish. After an obligatory latent period it could be demonstrated that Prausnitz developed a local reaction at the site of injection, every time he ate fish!

In the P.K. test, serum containing sensitizing antibody (IgE), is injected intradermally into a normal individual. The IgE fixes onto the local mast cells in the skin. After a latent period (allowed for IgE to fix to mast cells), antigen is administered intravenously with Evans blue. Blueing of the skin appears within minutes. This happens because antigen cross links the IgE molecules on the mast cell surface, causes degranulation and the resultant vascular permeability leads to blueing of the surrounding tissues. The P-K test demonstrates definitively, that IgE antibodies are responsible for cutaneous anaphylaxis and that this type of anaphylaxis can be passively transferred. The test is not done now for fear of transmitting AIDS or hepatitis B virus.

In active cutaneous anaphylaxis the animal is induced to produce IgE by antigen administration. In passive cutaneous anaphylaxis pre-made antibodies from another individual are used to elicit the reaction.

In vitro models for anaphylaxis

In vitro anaphylaxis was first demonstrated by Sir Henry Dale, in what is now known as the **Schultz - Dale reaction**, after its authors. Smooth muscle strips from ileum or uterus are bathed in physiologic buffered saline to which antibody from a hypersensitive animal is added. On addition of specific antigen, smooth muscle contractions occur in these bits of tissue in vitro. Bits of lung or skin can be used to demonstrate the same effect, which is due to degranulation of tissue mast cells and release of vaso-active amines.

In man, the term reaginic or skin sensitizing antibodies has been used synonymously with homocytotropic antibodies. The antibodies that accomplish this are most often IgE in all species.

The mechanisms involved in anaphylaxis

Following the first exposure to antigen, the animal responds by making antibody. Due to the nature of antigen (allergen) IgE is formed, which fixes onto mast cells via receptors that exist on mast cells for the Fc portion of the IgE molecule. The animal displays no symptoms but is now considered sensitized (Figure 15.1 a). On second exposure to the same antigen, molecules of antigenic material seek out the tissue fixed IgE antibodies and cross link adjacently placed IgE molecules (Figure 15.1 b). Bridging of adjacent Fab sites on the IgE molecules is rapidly followed by the breakdown of phosphatidyl inositol to inositol triphosphate (IP3), the generation of diacylglycerol and increase in intra cytoplasmic free calcium. These events activate protein kinase C. This familiar biochemical cascade produces membrane - active “fusogens” such as lysophosphatidic acid which facilitates degranulation and synthesis of arachidonic acid metabolites.

The **preformed or primary mediators** that are released from the granules are:

- histamine.
- heparin
- eosinophil chemotactic factor
  A for anaphylaxis (ECF-A)
- neutrophil chemotactic factor (NCF-A)
- platelet activating factor

The **newly synthesized** or secondary metabolites are :

- leukotrienes B4, C4, D4 and E4
  or slow reacting substance A (SRS-A)
- prostaglandins
- thromboxanes

Under normal circumstances, these mediators help orchestrate the development of a defensive acute inflammatory reaction. In a run away reaction their bronchoconstrictive and vasodilatory reactions can be life threatening.

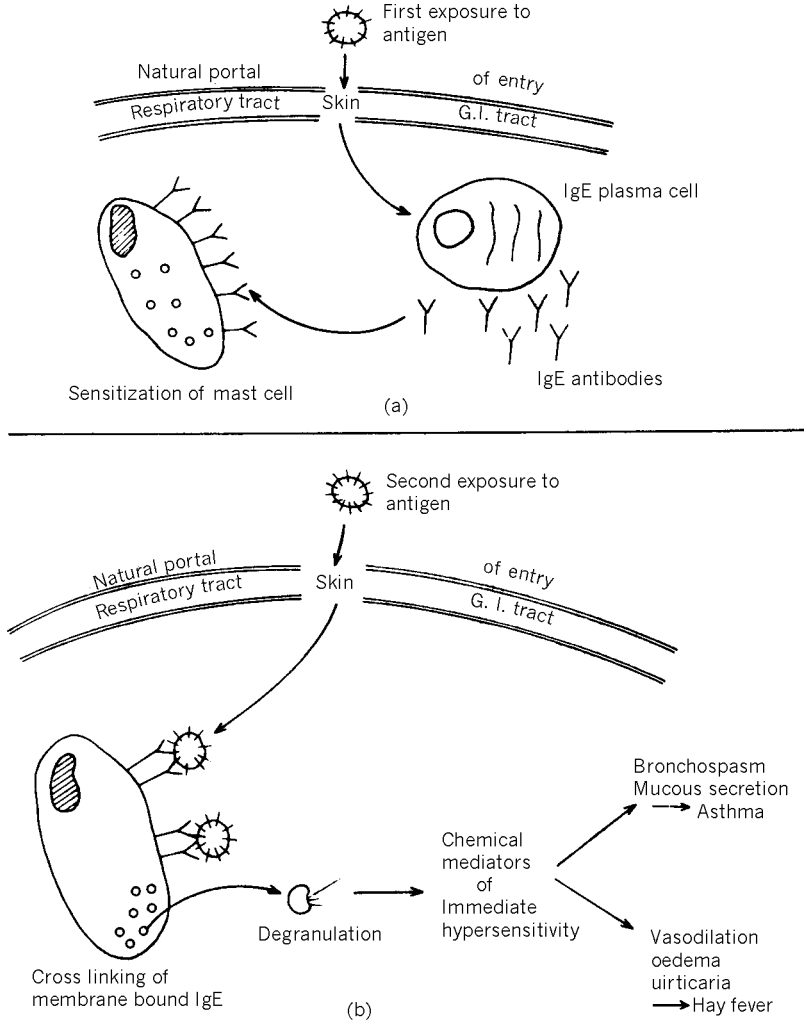


Figure 15.1. Mechanisms involved in anaphylaxis, (a) first exposure to antigen, (b) second exposure to antigen.

IgE receptors on mast cells

Mast cells and basophils have specific receptors that form non covalent bonds with the Fc portion of the IgE molecule (Figure 15.2). It is estimated that a basophil has 10,000 to 40,000 such receptors. The normal half life of IgE in serum is two to three days, however, IgE may remain fixed to cells for weeks. Heating IgE to 56° C for 30 minutes destroys its ability to bind to the target cell.

There are two types of IgE receptors. The high affinity IgE receptor on basophils and mast cells has been termed $Fc^{E}RI$; $Fc^{E}RII$ has low affinity for IgE and is found on a variety of leukocytes, including monocytes, macrophages, eosinophils, platelets and T and B cells. The high and low affinity receptors for IgE are antigenically distinct; they have different structures and are

encoded by separate genes. Fc^ERI is composed of three polypeptide chains, whereas Fc^ERII may play a major role in immunity against parasitic infections.

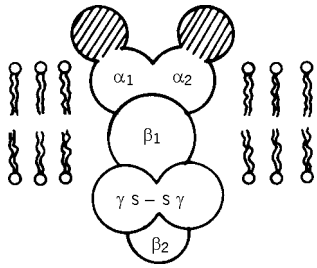


Figure 15.2. The Fc (IgE) receptor on the mast cell. The α chain binds the Fc portion of the immunoglobulin IgE. The b chain traverses the membrane and the covalently linked g chains face the cytoplasm.

Mediators of Immediate Hypersensitivity

Mast cells and basophils contain a number of preformed mediators with potent biologic activity. Mast cells contain a chymotrypsin like enzyme, chymase; basophils contain a kallikrein like esterase. Other mediators are generated after the mast cells are triggered (See Table 15.1).

Table 15.1: Chemical mediators of immediate hypersensitivity

Primary (pre-formed)		*Secondary (induced)*	
Mediator	***Functions***	***Mediator***	***Functions***
Histamine	Increased vascular permeability Elevation of cAMP Contraction of smooth muscle Chemokinesis	Leukotrienes: C4,D4,E4 or SRS-A	Contraction of human bronchiole, increased vascular permeability
ECF-A	Chemotactic for eosinophils	Platelet activating factor	Aggregation and increased secretion
NCF-A	Chemotactic for neutrophils	Lipid chemotactic, lipid chemokinetic factor	Neutrophil movement and activation
Heprin	Anticoagulant and anti-complement	Prostaglandin D2	Vasoactive smooth muscle action
Chymase	Proteolysis	Leukotriene B4	Increased vascular permeability; eosinophil, neutrophil chemotaxis, neutrophil adhesion
		Thromboxanes	Aggregate platelets

When released, the mediators can cause increased vascular permeability increased secretion by nasal and bronchial mucous glands and contraction of smooth muscle in bronchioles and small blood vessels. Eosinophils and neutrophils are summoned to the area of injury and bradykinin is generated by the kallikrein-like esterase. The increased capillary permeability leads to an influx of plasma proteins including antibody, complement and kinin generating and

coagulation proteins into the tissues. All of these substances increase inflammation. The clinical manifestations depend on the sites affected and include systemic anaphylaxis, urticaria, asthma, rhinitis and vasculitis.

A major group of secondary mediators, the leukotrienes, are potent bronchoconstrictors and vasodilators (See Table:15.1). Leukotrienes are formed after the enzyme lipoxygenase acts on arachidonic acid. Also derived from arachidonic acid, by the action of cyclooxygenase, are prostaglandins which perform related inflammatory functions.

Regulatory Mechanisms of Immediate Hypersensitivity

Cross linking of IgE on mast cells results in explosive degranulation of the cell. Activation of enzymes for degranulation involves a complex series of biochemical steps including transmethylation of membrane phospholipids, activation of protein kinases and adenylate cyclase, changes in intracellular cAMP levels and the opening of Ca++ channels. This process is regulated at several levels:

(*i*) The intensity of the reaction depends on the amount of IgE bound to the cells, the affinity and concentration of antigen for IgE. If IgG has occupied a majority of sites on the mast cell, cross linking of IgE does not occur and degranulation is prevented. Such IgG molecules are called **blocking antibodies**.

(*ii*) Mediator release is influenced by intracellular cyclic nucleotides. Agents that induce prolonged elevations of intra cellular cAMP lead to decrease in mediator release; agents that increase cyclic GMP have the opposite effect. These agents act via receptors on the cell surface (Figure 15.3).

(*iii*) Histamine can exert negative feed back control by stimulating adenylate cyclase which increases cyclic AMP and thereby prevents further release of histamine.

(*iv*) Eosinophils attracted to the site by chemotactic factors are also thought to have a control function. They contain histaminase which breaks down histamine and phospholipase D, which acts on platelet activating factor. Enzymes from neutrophils also break down mediators.

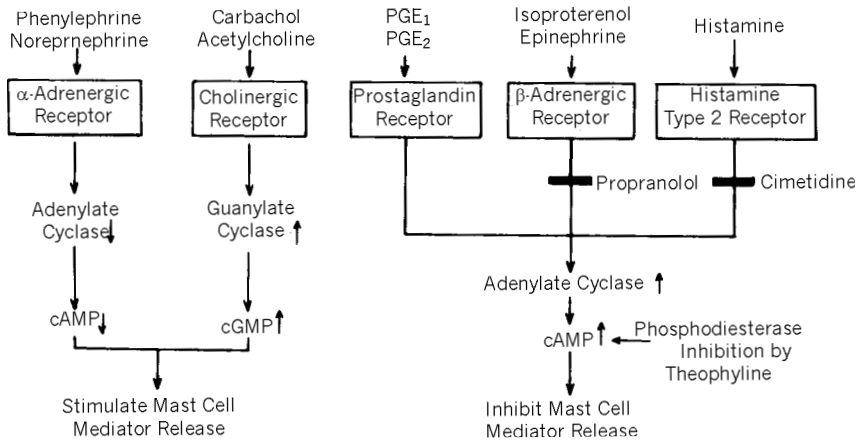


Figure 15.3. Mediator release by mast cells is modulated by the cyclic nucleotides cAMP and cGMP. Decreased cAMP or increased cGMP levels stimulate release, whereas increased cAMP inhibits release. A number of physiologically active substances can trigger cell surface receptors, affecting cyclic nucleotide production and, secondarily, mast cell release of mediators.

Atopic allergy

Nearly 10% of the world's population suffer from allergies, which are localized anaphylactic reactions to extrinsic antigens such as pollens, foods, animal danders and faeces from mites in house dust to name just a few. Contact of the allergen with cell bound IgE in the bronchial tree, the nasal mucosa, skin and the conjunctival tissues releases mediators of anaphylaxis and produces the symptoms of hay fever, asthma, urticaria or allergic rhinitis as the case may be. Contact with food allergens and cell bound IgE in the gastrointestinal tract may cause diarrhoea and vomiting. Allergen-IgE complex may diffuse out of an already permeable gut and deposit in joints, skin or lung causing further local anaphylactic reactions. Thus eating prawns or egg may precipitate an asthmatic attack in a sensitized individual.

There is a strong familial disposition to the development of atopic allergy, and this may be linked to the inheritance of specific HLA haplotypes, though culprit HLA types have not yet been identified. It has been shown that the higher the level of serum IgE, greater is the likelihood of becoming atopic.

Clinical tests for allergy

Sensitivity is normally assessed by the response to intradermal administration of allergen. The release of histamine and other mediators produces a wheal and flare reaction in 30 minutes. The immediate wheal and flare reaction may be followed by a late phase reaction which sometimes lasts for 24 hours. This reaction is thought to be mediated by NCF-A following cellular infiltration into the area.

Other tests include the **RIST** and the **RAST** (described in Chapter 8).

Therapy for atopic diseases takes advantage of some of the regulatory mechanisms of immediate hypersensitivity mentioned earlier (Figure 15.4). Examples include, diminishing antigen exposure, inducing blocking IgG antibodies, use of mast cell stabilizers such as sodium cromoglycate; therapy with catecholamines such as epinephrine and isoproterenol to elevate cAMP and decrease mediator release and the administration of antihistamines to block the effect of histamine on target organs. It may be noteworthy that both propranolol and cimetidine may induce allergic manifestations in susceptible individuals by lowering cAMP and thereby promoting mast cell mediator release.

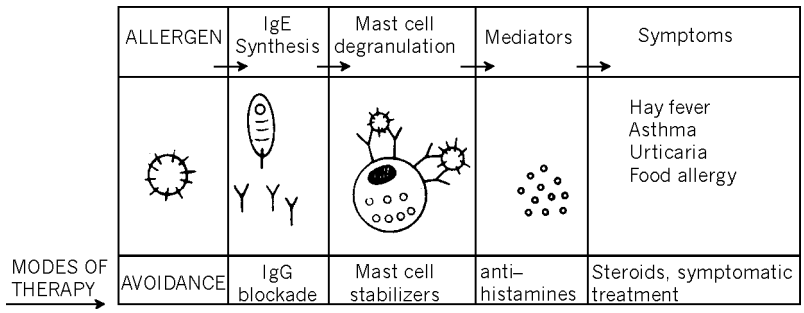


Figure 15.4. Possible modes of therapy for atopic diseases.

A new experimental approach to treating and preventing Type-I hypersensitivity involves giving the person with allergies injections of **monoclonal antibodies** that have been made **against the Fc portion of human IgE**. This, in turn, blocks the attachment of the IgE to the

Fc receptors on mast cells and basophils and the subsequent release of histamine by those cells upon exposure to allergen. In addition, the anti-IgE binds to IgE-producing B-lymphocytes causing apoptosis. The monoclonal antibody is a humanized hybrid molecule consisting of a mouse binding (Fab) portion attached to a human constant (Fc) portion and is known as rhuMab (recombinant human monoclonal antibody).

Type II-Antibody Dependant Cytotoxic Hypersensitivity

The combination of IgM or IgG antibodies with antigenic determinants on the cell surface renders the cell surface coated or opsonized by antibody. Such an opsonized particle is readily phagocytosed by macrophages which have Fc receptors for IgG or IgM molecules on their surface (Figure 15.5a). The antibody facilitates the association between the phagocytic cell and the antigen bearing cell. If this happens in excess and causes tissue damage it is one example of the antibody dependant cytotoxic hypersensitivity.

There are several other mechanisms which account for type II hypersensitivity. These include opsonization via C3b receptors which also exist on surfaces of macrophages (see Chapter 7) and resultant phagocytosis (Figure 15.5b). Excessive cell lysis may occur when cell surface antigen couples to antibody, fixes complement and activates the entire complement cascade (Figure 15.5c).

Another quite distinct mechanism in type II hypersensitivity is that mediated by K cells, in what is known as antibody dependant cellular cytotoxicity-ADCC (see Chapter 14), (Figure 15.5d). It is quite evident thus far that type II hypersensitivity may or may not involve the participation of complement.

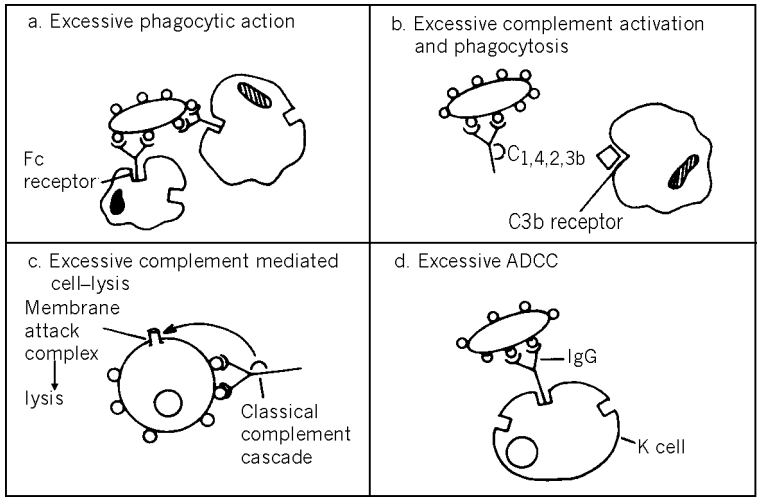


Figure 15.5. Mechanisms of antibody dependant cytotoxic hypersensitivity.

Type II reactions in blood transfusion and organ transplantation

The basis for ABO blood grouping

Of the many variant antigens on the human red cell membrane, the ABO antigens form the dominant system. Individuals with blood group O display the H substance on the red cell

surface (Figure 15.6). The H substance is an oligosaccharide anchored to the cell membrane by coupling to a sphingomyelin called ceramide. This oligosaccharide has a terminal galactose residue coupled to a fucose at position 2. Individuals who are blood group A produce certain glucosyl transferases encoded by an A gene. These enzymes act by adding N acetyl galactosamine to position 3 of the terminal galactose of the H substance. Similarly individuals who are blood group B posses enzymes encoded by the B gene which couple another galactose to position 3 of the galactose residue in the H substance. Individuals belonging to the AB blood groups possess both genes and hence both antigens on the red cell surface.

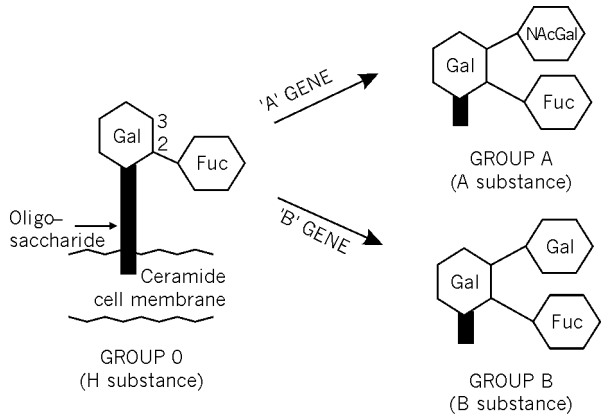


Figure 15.6. The basis of the ABO Blood Group System.

Naturally occurring antibodies or **isohaemagglutinins** in individuals are targeted to those antigens absent from the red cell surface. Thus a person with blood group A possesses anti B antibodies and vice versa. Persons who are blood group AB have neither anti A nor anti B antibodies and individuals with blood group O, present with both anti A and anti B antibodies. These isohaemagglutinins are usually IgM. Since they are seemingly present in the circulation, without antigen provocation, how were they induced? It has been thought that bacterial antigens of the normal gut flora cross react with blood group antigens. Hence, if an individual is blood group A, he will be tolerant to antigens closely similar to A (see theories of tolerance, Chapter 16) and will form antibodies to bacterial antigens that cross react with blood group B. Similarly, an individual with blood group O, is not tolerant to either antigen and therefore will produce antibodies to substance A and B. Persons with blood group AB are tolerant to both antigens and hence do not produce the appropriate antibodies.

Mismatched Transfusion

On transfusion of mismatched red cells, the donor red cells are rapidly coated with the host's isohaemagglutinins and severe reactions ensue, which utilize complement. Since IgM is involved, cross linking of just a few determinants is sufficient to set off the entire complement cascade (Figure 15.7). Runaway complement activation with the resultant tissue reaction is a classic example of a type II hypersensitivity reaction.

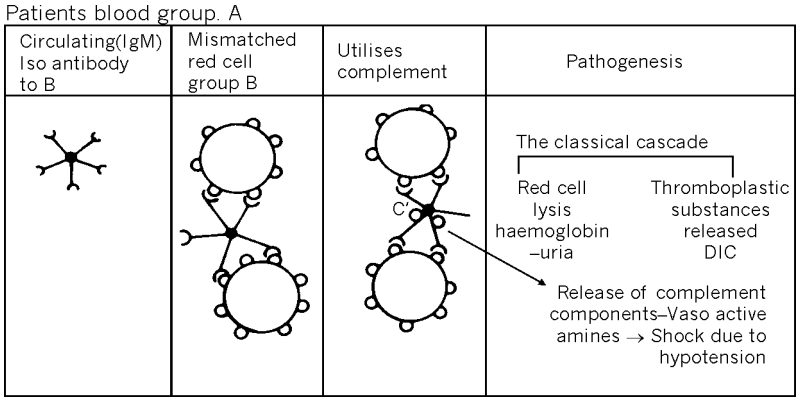


Figure 15.7. Mismatched transfusion reactions DIC = Disseminated intravascular coagulation.

Rhesus incompatibility

The rhesus blood groups form another major antigenic system, the rhesus antigen is designated RhD or just D. Antibodies to RhD, unlike the isohaemagglutinins, are not naturally occurring. An individual who does not display RhD on his red cell surface (RhD antigen negative), needs to be exposed to the D antigen to be able to produce anti D antibodies. An individual who has the RhD antigen on his red cell membranes, is of course tolerant to the antigen and does not produce anti D antibodies. A mother who is RhD negative may give birth to an RhD positive child (the RhD antigen being inherited from the father). On delivery of the first child, during separation of placenta, there is some release and admixing of foetal red cells in the maternal circulation. The mother being exposed to the RhD antigen is sensitized and begins to produce anti D antibodies. These antibodies are predominantly IgG and are able to cross the placenta in any subsequent pregnancy. This IgG reacts with foetal red cells, during subsequent pregnancies, leading to foetal red cell destruction in utero, through another type II mediated hypersensitivity reaction, resulting in haemolytic disease of the newborn. Foetal red cells coated with maternal anti D IgG and circulating anti D IgG in the maternal blood can be detected in the laboratory by the Coomb's direct and indirect tests (see Chapter:8).

To prevent haemolytic disease of the newborn, RhD negative mothers are given passive immunization with small amounts of ready made anti D IgG antibodies at the time of birth of the first child. These small amounts of anti D IgG will complex with RhD on those foetal red cells that escape into the maternal circulation during placental separation. This precludes the presentation of foetal RhD antigen to the mother's immune system and sensitization of the mother to RhD is prevented.

Organ transplants

A long standing homograft may evoke antibodies in the host which are directed against surface antigens on the transplanted tissue. These may be directly cytotoxic or cause adherence of phagocytic cells. They may also elicit non specific attack by K cells via ADCC. The antibodies may lead to platelet adherence, when they are directed against antigens on vascular endothelium. All of these reactions are examples of Type II mediated hypersensitivity reactions.

Autoimmune Type II Hypersensitivity

Many type II hypersensitivity reactions are the underlying mechanisms of autoimmune disease. Auto antibodies to the patient's own red cells are produced in autoimmune haemolytic anaemia. Red cells coated with bacterial or viral antigens cause antibodies to be directed against them, with resultant red cell lysis.

Sera of patients with Hashimotos thyroiditis contain antibodies, which in the presence of complement, are directly cytotoxic for isolated human thyroid cells in culture. In Goodpastures syndrome, auto antibodies are directed to glomerular basement membrane; they fix complement and serious damage ensues. Antibodies to acetylcholine receptors in myasthenia gravis is a further example of type II hypersensitivity. In multiple sclerosis antibodies are made against the oligodendroglial cells that make myelin in the brain and spinal cord.

Type II Hypersensitivity due to drugs

Drugs coupled to the body's own cells become antigenic and the humoral immune system recognizes the drug-cell complex as foreign, resulting in an antibody attack which destroys the cells as well. When the drug is withdrawn, the sensitivity is no longer evident. With drugs such as chlorpromazine and phenacetin, red cells become coupling agents and a type II haemolytic anaemia is seen. Amidopyrine and quinidine are known to coat granulocytes and a dreaded agranulocytosis could result. The classic example of drug induced type II hypersensitivity, is that of thrombocytopenic purpura, when sedormid, a now banned sedative, known to bind to platelets was used. This reaction, too, was shown to be facilitated by complement.

Type III-Immune Complex Mediated Hypersensitivity

Pathogenesis of immune complex disease

The term immune complex disease refers to a group of diseases whose pathogenesis involves tissue damage from excessive antigen-antibody reactions. The body may be exposed to excessive amounts of antigen in a number of circumstances, such as persistent infection with microbial agents, autoimmune reactions and repeated contact with environmental agents. When antigen and antibody couple to form insoluble complexes at fixed sites, tissue reaction and tissue damage could well occur. If complement is involved, C3a and C5a, two potent vasoactive amines, are released; this causes increased vascular permeability. Increased vascular permeability is a pre requisite for the deposition of immune complexes in tissues. If antigen in the circulating complex reacts with IgE on circulating basophils, it causes platelet clumping forming microthrombi, degranulation and the subsequent release of histamine, serotonin and other chemotactic factors. Chemotactic factors lead to influx of polymorphonuclear leukocytes. This in turn leads to extracellular release of polymorph granular contents. These include proteolytic enzymes, kinin forming enzymes and other proteins which will damage tissues and intensify the inflammatory process. Large insoluble complexes taken up by macrophages cannot be readily digested and provide a persistent activating stimulus (Figure 15.8)

Clearance of immune complexes in vivo normally occurs, otherwise we would all be suffering from immune complex disease! Clearance depends on the absolute amounts of antigen and antibody which could determine reaction intensity. However, proportions of antigen and antibody also govern the nature of complexes. Extreme antibody excess or antigen excess does not yield insoluble precipitated complexes. An optimum ratio of antigen to antibody provides for an insoluble complex (lattice), (Figure 8.2). Complement fixation influences size and solubility

of immune complexes (Figure: 15.9). Binding of complement component, C1, to immunoglobulin prevents Fc-Fc interactions needed to form large insoluble aggregates. Smaller complexes which have C3b attached to them are cleared away by macrophages which have C3b receptors on their surface. This clearing away is aided by red cells bearing the CR1 receptor which couples to C3b in the antigen - antibody - C3b complex and transports it away to fixed macrophages in the liver where they are inactivated.

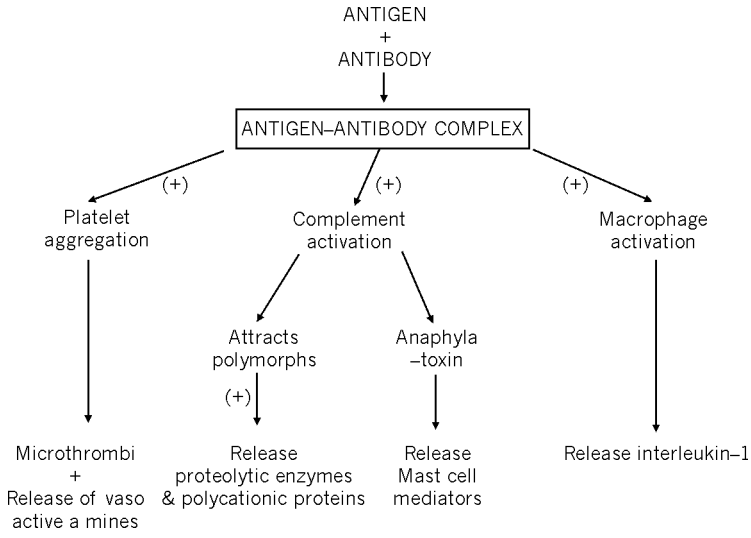


Figure 15.8. Schematic representation of Type III immune-complex mediated hypersensitivity.
(+) = stimulation of process.

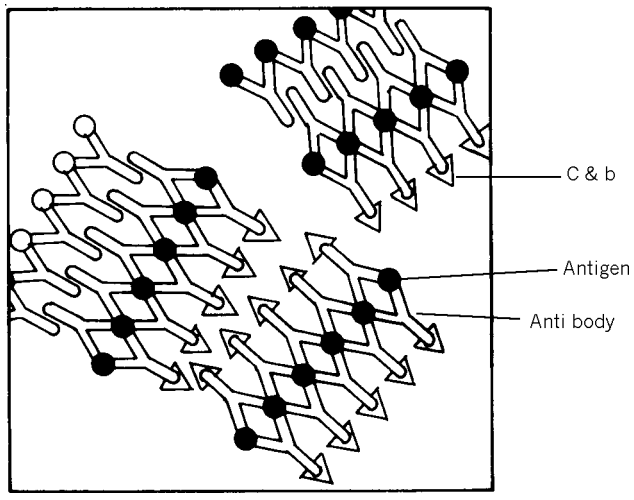


Figure 15.9. An immune complex lattice can be disrupted by complement component C3b, which weakens the forces between the Fc portion of the antibody molecules. This disruption solubilizes the lattice and facilitates clearance of immune complexes from the circulation.

Where are immune complexes deposited?

When large insoluble complexes form, or when the clearing system is deficient, immune complexes are deposited in tissues with resultant damage. Deposition can occur anywhere in the body, but some organs such as the kidney are affected more often than others. The combination of high blood flow, rapid filtration and high blood pressure in renal glomeruli facilitates deposition of immune complexes. Receptors for complement components are found in the glomeruli and in the choroid plexus - another favoured site for deposition of immune complexes. In addition, basement membranes, whether in the glomerulus or in the dermal-epidermal junction of the skin, are negatively charged and will therefore retain positively charged immune complexes.

When the ability of red blood cells to act as carriers of immune complexes is impaired, complexes are more likely to be deposited in blood vessels. Such an impairment occurs in patients who have systemic lupus erythematosus, a disease in which red cells have decreased C3b receptors. Likewise, if scavenger systems in the liver or spleen are impaired, immune complexes continue to circulate and deposit in other organs.

Local reactions to Type III mediated Immune Complex Deposition

The Arthus Reaction

The Arthus reaction was described by Maurice Arthus who found that an acute haemorrhagic or necrotic process occurs locally, when antigen is injected into immunized individuals possessing high titres of precipitating antibody. This reaction is considered the prototype acute, localized immune complex mediated tissue injury. The injected antigen precipitates with antibody, often within a venule; subsequently, the complex binds complement component C1q, thereby activating the classical complement pathway. The reaction generates proteolytic cleavage products such as C3a, C3b and C5a. C3a and C5a cause mast cells to release vasoactive amines. Intravascular complexes cause platelet aggregation and further release of vasoactive amines from basophils. The end result of all this is severe erythema and oedema. C5a is a powerful chemo attractant and polymorph influx results. Polymorphs release a wide variety of products (see earlier section) that degrade basement membrane and cause tissue damage. The Arthus reaction is thus primarily mediated by complement activation and neutrophil released products. The Arthus reaction can be blocked by depletion of complement and neutrophils.

Type III Reactions to inhaled antigens

Arthus type reactions in the lung can occur to a variety of inhaled antigens. A classic example is Farmers lung, which occurs 6-8 hours after exposure to mouldy hay. These patients have been found to be sensitized to thermophilic actinomycetes which grow in mouldy hay. Inhalation of the organism from the hay, introduces antigen into the lung and a immune complex mediated hypersensitivity reaction occurs. Similar reactions can occur from bird and rodent excreta in bird fanciers disease and in rat handlers disease respectively. There are many other quaintly named conditions of similar nature due to extrinsic allergic alveolitis resulting from inhalation of foreign antigens.

Type III Reactions to tissue antigens

Type III reactions are associated with elephantiasis due to *Wuchereria bancrofti*. The dead parasite, found in lymphatic vessels, initiates a type III reaction causing inflammation and obstruction to lymph flow, severe fibrosis and resultant elephantiasis.

Vigorous chemotherapy is known to cause reactions due to abrupt release of microbial antigens in patients with high antibody levels. Dramatic immune complex mediated reactions manifest as erythema nodosum leprosum in dapsone treated cases of lepromatous leprosy and in the Jarisch - Herxheimer reaction of syphilis patients treated with penicillin. In rheumatoid arthritis, antibodies to self IgG get deposited in the joint contributing to severe synovitis.

Type III mediated Systemic Immune Complex Disease

Serum sickness was first described early in the century in patients who received horse serum for the treatment of infectious diseases. Typically, 8-12 days after the administration of large amounts of serum from a foreign species, the patient may have a rise in temperature, tender lymph nodes, painful and swollen joints, an urticarial rash, gastrointestinal distress and an enlarged spleen. This is associated with lowered serum complement levels and leukocytosis. These changes result from deposition of immune complexes in tissues. To be pathogenic, immune complexes have to be the right size; not too big to be scavenged by macrophages and not too small, so as not to cause an inflammatory reaction. The complexes lodge in blood vessels and cause changes in vascular permeability. This results in separation of capillary endothelial cells and exposure of the underlying basement membrane to which the immune complexes attach. Basement membranes of skin, joints, kidneys and the heart are particularly affected.

In addition to foreign serum, several drugs can cause serum sickness, including penicillin, gold, penicillamine, sulfonamides, hydantoins, thiazides, phenylbutazone, aminosalicylic acid and streptomycin. These drugs act as haptens, complex with protein carriers and induce antibody formation. Repeated administration of foreign proteins and drugs with repository or long acting characteristics, such as penicillamine or gold, may induce chronic serum sickness. The major complications of serum sickness are vasculitis, glomerulonephritis and neuritis.

Immune Complex Nephritis

Because renal glomeruli are particularly vulnerable to immune complex-mediated injury, glomerulonephritis is a common feature of many immune complex diseases. Complexes form in the circulation and if they are large enough, they are deposited in the endothelial side of the glomerular basement membrane (Figure 15.10). The smallest complexes manage to filter through to the epithelial side. The depositions of immune complexes appear as lumpy granules and contain antigen, immunoglobulin and complement; they are visible as large amorphous masses by electron microscopy. The pathogenesis of immune complex mediated glomerulonephritis is illustrated in Figure 15.10.

Glomerulonephritis has been associated with persistent **bacterial** products. Glomerulonephritis is well known as a sequela to infection with type 12 β haemolytic, “nephritogenic”, Streptococci. Glomerulonephritis can also occur after pneumococcal and staphylococcal infections.

Viral infections with hepatitis B and Epstein-Barr virus are associated with immune complex disease. Antigenic similarity between human and microbial antigens has been hypothesized as being the cause of vigorous antibody formation against host tissues and resultant immune complex deposition.

Immune complex nephritis has been associated with **parasitic** disease. Quartan malaria in some Nigerian children leads to an immune complex mediated nephrotic syndrome. Chronic parasitoses such as leishmaniasis, trypanosomiasis, schistosomiasis and filaria are particularly associated with chronic glomerulonephritis. Chronic stimulation of the immune system and polyclonal B cell stimulation has been incriminated as possible aetiological factors in these conditions.

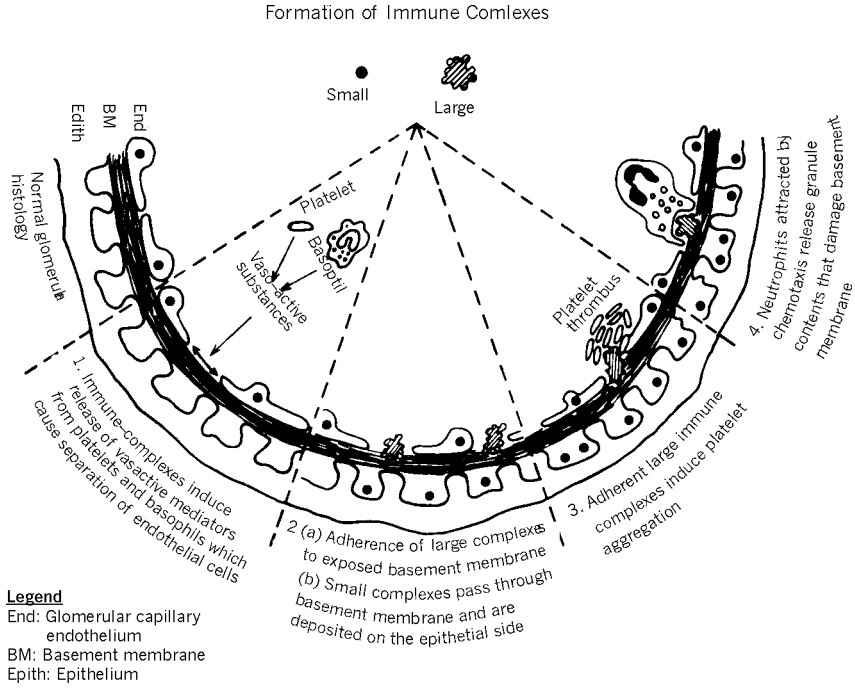


Figure 15.10. Pathogenesis of Immune-complex Nephritis.

Systemic lupus erythematosus (SLE), an **autoimmune** disease is the paradigm immune complex disease. Immune complexes have been found in kidneys, blood vessels, skin and the central nervous system. Immune complexes isolated from lesions in SLE contain DNA and antibody to DNA. Vigorous deposition of DNA-anti DNA complexes is explained, in part, by the finding that there are fewer CRI receptors on red cells and monocytes in these patients and hence immune complexes cannot be transported away to the reticulo endothelial system.

Immune complex nephropathy can be found in **malignant** disease such as lymphocytic leukaemias and Hodgkins disease. **Gastrointestinal disease** such Crohns disease, ulcerative colitis, cirrhosis of the liver and coeliac disease can be associated with immune complex tissue injury. Deposition of immune complexes can occur at other major filtration sites such as the choroid plexus. Deposition also occurs at the basement membrane of the dermal - epidermal junction, and the endothelial linings of blood vessels.

Detection of circulating immune complexes

Tissue bound complexes are usually visualized using immunofluorescent staining. A multitude of assays exists for the detection of circulating immune complexes.

One assay is based on the tendency of complexes to precipitate in the cold. The **cryoprecipitates** are then analysed for immuno globulin type, antigen content and presence of complement.

A more sensitive assay is based on the ability of immune complexes to bind C1q. C1q is radiolabelled and mixed with patient's serum. The immune complexes bound to C1q are precipitated out, the amount of unbound C1q is quantified as an indirect indicator of presence of immune complexes.

Other assays depend on the ability of immune complexes to bind to cells that have receptors for the Fc portion of IgG or for complement components, such as the Raji cell line or macrophage cell lines. The assays measure the amount of immune complexes bound to cells. Close monitoring of circulating immune complex levels helps to evaluate therapy in a number of disease states.

Type IV-Cell Mediated or Delayed Type Hypersensitivity

Delayed hypersensitivity is a cell mediated immune reaction in an individual previously sensitized to an antigen. A skin reaction typically develops 12-48 hours after intradermal injection of antigen. The best known example of this reaction is the positive Mantoux reaction where tuberculin is injected into the skin of an individual, in whom previous exposure to *Mycobacterium tuberculosis* has induced a state of cell mediated immunity (CMI). The reaction appears after several hours and may take upto 48 hours to reach a maximum, hence the usage of the term "delayed" hypersensitivity. Histologically, the lesion consists of a predominantly mononuclear cell infiltrate - of the monocyte- macrophage series. This contrasts with the essentially neutrophil predominant character of the Arthus reaction.

Similar lesions can be seen in organs undergoing cell mediated immune reactions. For example, the granulomas that form around *Schistosoma mansoni* eggs in the liver, are the result of cell mediated immune reactions. Cell mediated hypersensitivity is responsible for the cavitation and caseation of tubercular lesions and for the granulomatous skin lesions of the borderline form of leprosy.

The skin rashes of small pox and measles and the lesions of herpes simplex have been attributed to delayed type hypersensitivity reactions with associated cytotoxic T cell damage to virally infected cells. Cell mediated hypersensitivity is also evident in fungal diseases such as candidiasis, dermatomycosis, coccidioidomycosis and histoplasmosis and in parasitoses such as leishmaniasis and schistosomiasis.

Cellular Reactions in type IV-Hypersensitivity : the chronic granuloma

The type IV hypersensitivity reaction results from an exaggerated interaction between antigen and a normal cell mediated immune reaction. Delayed hypersensitivity therefore has the same mechanism as cell-mediated immunity. T cells recognize antigen together with MHC Class II molecules and are activated, resulting in proliferation and release of lymphokines. T8-lymphocytes once sensitized to an antigen differentiate into cytotoxic T-lymphocytes while T_h1 type T4-lymphocytes become sensitized to an antigen and produce cytokines . When this reaction does not get rid of the offending antigen, persisting antigen evokes a chronic cell mediated hypersensitivity reaction. CTLs, cytokines, and/or macrophages then cause harm rather than benefit. Continual release of lymphokines leads to infiltration of macrophages with arrays of epithelioid cells. Some cells fuse to form multinucleated giant cells. Further, tissue damage occurs when K cells and NK cells join the foray against foreign antigen and indiscriminate cytotoxicity results. Histologically, there is evidence of lymphocytes, macrophages, epithelioid cells and giant cells. Tissue necrosis is surrounded by the above cellular reaction with areas of fibrosis in the periphery. This lesion represents the chronic granuloma and is an attempt by the body to wall off a site of persistent infection.

Unlike other forms of hypersensitivity, delayed hypersensitivity does not involve antibody and cannot be transferred from a sensitized individual to a non sensitized individual with serum antibody.

Cutaneous Basophil Hypersensitivity

Cutaneous basophil hypersensitivity is a term for a group of delayed onset lymphocyte mediated reactions which have been studied extensively in guinea pigs. They are also seen in humans and were originally called **Jones - Mote** reactions. They differ from classic delayed hypersensitivity reactions in a number of ways. The skin lesions are intensely infiltrated by basophils as well as lymphocytes, they are erythematous but lack the induration and fibrin deposits in classic delayed type reactions. This type of reaction is seen in human contact dermatitis and in skin and renal allograft rejection. At present it is not known what factors determine whether a reaction will terminate as a basophil hypersensitivity or a classical delayed type hypersensitivity reaction.

Contact Hypersensitivity (contact dermatitis)

Contact hypersensitivity can occur in people who become sensitized while working with chemicals such as picryl chloride and chromates, or who repeatedly come into contact with poison ivy. p-Phenylene diamine in certain hair dyes, neomycin in topically applied ointments and nickel salts as in nickel coated costume jewellery can evoke a similar reaction. These chemical substances bind to body constituents to form antigenic material capable of inducing a delayed hypersensitivity reaction. The reaction to these neo-antigens is characterized by a mononuclear infiltrate, accompanied by oedema of the epidermis and micro vesicle formation.

A summary of the different types of hypersensitivity is presented in Table: 15.2

Table 15.2: Comparison of different types of hypersensitivity

Type	*Descriptive Name*	*Initiation Time*	*Mechanism*	*Examples*
I.	IgE-mediated hypersensitivity	2-30 mins	Ag induces cross – linking of IgE bound to mast cells with release of vasoactive mediators	Systemic anaphylaxis, local anaphylaxis, hay fever, asthma, eczema
II.	Antibody-mediated cytotoxic hypersensitivity	5-8hrs	Ab directed against cell-surface antigens mediates cell destruction via ADCC or complement	Blood transfusion reactions, haemolytic disease of the newborn, autoimmune haemolytic anaemia
III.	Immune-complex mediated hypersensitivity	2-8hrs	Ag-Ab complexes deposited at various sites induces mast cell degranulation via Fcgamma, PMN degranulation damages tissue	Arthus reaction (Localised); systemic reactions disseminated rash, arthritis, glomerulonephritis
IV.	Cell-mediated hypersensitivity	24-72hrs	Memory T_h1 cells release cytokines that recruit and activate macrophages	Contact dermatitis, tubercular lesions

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CHAPTER – 16

IMMUNOLOGIC TOLERANCE AND AUTOIMMUNITY

One of the central concepts of immunology has been that the immune system should be able to distinguish between "self" and "non self". Immune reactions to "self" antigens constitutes what is known as autoimmunity and is injurious to the host. Around 1900, Paul Ehrlich postulated that the immune system acquires a state of tolerance to self antigens, in addition he proposed that break down of tolerance would lead to self destruction, a condition he described as "horror autotoxicus". Over 40 years ago, Owen demonstrated tolerance in non identical (dizygotic) twin calves who shared the same placental circulation. Even though each calf had appreciable numbers of red cells from the other twin (due to the common placenta), they did not mount a reaction to the other's red cells. If they had not shared the same placental circulation, an infusion of the other twin's red cells in adult life, would have caused a severe immunological reaction.

About 50 years after Paul Ehrlich, Macfarlane Burnet suggested that immune cells reacting with antigens during development of the foetus are destroyed by lymphoid organs. This leads to tolerance to such antigens since the clones of cells reacting against such antigens have been deleted during foetal development. In the last 15 to 25 years there have been major revisions of these classic concepts. It has become clear that at a finer level of detail, the law that a normally functioning immune system does not recognize self is not absolute. The receptors of the immune system that perform the work of recognition can themselves be recognized by other receptors. Such "self recognition", which was strictly outlawed in the early years, may form the basis of a network whose equilibrium keeps the body healthy. Another important form of self recognition involves immuno competent cells which have been schooled to recognize the body's own major histocompatibility antigens. As discussed earlier, this form of self recognition forms the key reaction in many immunological processes beneficial to the host. Autoimmune disease occurs when these normal "autoimmune" reactions are disturbed.

Forms of Normal Auto Recognition or Positive Autoimmunity

The idiotype - anti idiotype network

It is now clear that the hypervariable regions of the immunoglobulin molecules react with antigenic determinants also called epitopes. Important discoveries clearly illustrate that those sites on the hypervariable region of the immunoglobulin molecule that bind to antigens are themselves immunogenic: being complex protein molecules. Individual antigenic sites on the immunoglobulin hypervariable region are called idiotopes. A set of idiotopes on a single immunoglobulin molecule is called the idiotype. If an idiotype is involved in antigen binding, it is called a paratope. It stands to reason that if an antigen such as the idiotype exists, then the human immune system must be equipped to produce antibodies to idiotypes. Hence it is now

clear that a network exists wherein the immune system produces anti idiotypic antibodies to its own idiotypes. Credit for these discoveries goes to Niels Jerne who in 1974, proposed that normal autoimmune responses to self idiotypes, might form the basis of immuno regulatory network systems. Homeostasis of the immune system is thus thought to be preserved through a functional assembly of idiotype anti idiotype interactions.

According to this model, an antigen induces production of an antibody (Ab1) characterized by its idiotype (Id1). In turn, (Id1) stimulates the synthesis of an anti idiotypic antibody (anti-Id1 or Ab2), bearing the idiotype Id2, that can, in turn, trigger the production of anti Id2 or (Ab3) (Figure 16.1). Theoretically, these idiotype - anti idiotype reactions can continue indefinitely. However, the interactions appear to be limited. In addition, it has been postulated that anti idiotypes that are directed towards paratopes (antigen combining sites), must stereochemically (by virtue of 3-D structure), resemble that part of the antigen that locks with the paratope. This is a reasonable postulate since both the anti idiotype and the antigen combine with the same binding site on the paratope. Such anti idiotypes were termed the internal image set by Jerne; Lindenmann called them homobodies.

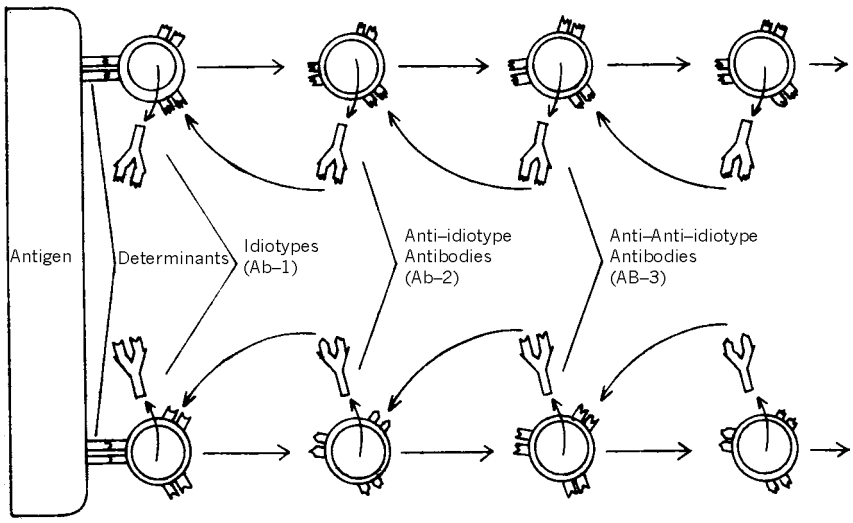

Figure 16.1. Idiotype-Anti idiotype reactions. The idiotype network has been proposed as a mechanism to regulate the immune response. The presence of a foreign antigen leads to production of antibodies (Ab-1) that recognize the determinants of that antigen. Each of these antibodies contains a collection of unique regions that are collectively known as the idiotype; the idiotype includes the antigen binding site of the immunoglobulin molecule. The presence of Ab-1 leads to production of a second type of antibody, anti-idiotype antibody (Ab-2), which recognizes the idiotypes of Ab-1. Ab-2 also contains idiotypes and hence leads to production of a third type of antibody, anti-anti-idiotype antibody (Ab-3). Although the network theoretically can continue indefinitely, it appears to be limited.

It is generally believed that the idiotype anti idiotype network contributes to the regulation of the immune system. Besides directly interacting with B cell receptors or with antibodies in the serum, anti idiotype antibodies could modulate the immune response by activating or repressing different T cell subsets. The concept emerging, is that idiotype - antiidiotype circuits may regulate immune responses at appropriate levels: the helper T cell stage, the plasma cell and the level of the formed antibody secreted into the circulation.

Recognition of Self MHC by T cells

This has been discussed extensively in earlier chapters. Suffice it to say, that it has now been demonstrated beyond any doubt that immunocompetent cells of the vertebrate immune system preserve and express through out life, a self recognition capacity especially of MHC molecules. This is now known to be essential for the normal functioning of the immune system in all its diversity.

Tolerance

If the normal immune system can respond to virtually any foreign substance, why does it not respond destructively to self antigens? The absence of harmful immune reactions to antigens is termed tolerance. It has been shown that tolerance can be induced in animals by varying the dosage of the immunogen. Rabbits which are repeatedly exposed to low doses of a weak immunogen, remain tolerant even to a strongly immunogenic form of the same antigen. Similarly, tolerance can be induced even if high doses of the immunogen are administered to the animal. Hence there seems to be a **low zone** and a **high zone** in terms of antigen dosage for tolerance induction (Figure 16.2). Experiments have shown that the T cell is the target for low zone antigen tolerance and both T cells and B cells are irresponsive at high antigen dosage.

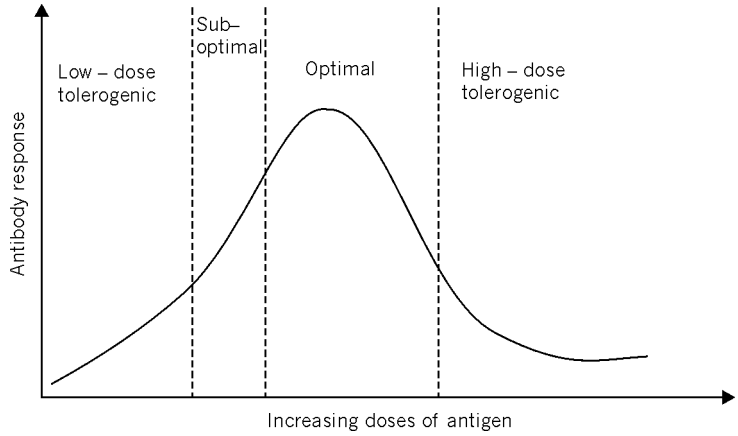

Figure 16.2. High zone and low zone tolerance.

How does an individual tolerate self antigens while generating normal immune reactions to foreign antigens? The answer to this question is not known. Studies indicate that there are several mechanisms which may contribute to tolerance: clonal deletion, clonal anergy, receptor blockade, non- immunogenic antigens, anti idiotype antibodies, suppressor T cell factors and other regulatory factors.

Theories of Tolerance Induction

We can divide the mechanisms the immune system uses to ensure the absence of self-reactivity (**autoimmunity**) into two main types:

- **Central Tolerance**: this occurs during lymphocyte development.
- **Peripheral Tolerance**: occurs after lymphocytes leave the primary organs.

Central Tolerance

Macfarlane Burnet proposed the theory of **clonal selection** (Refer Chapter 11). In a brief recapitulation, the theory states that clones of immune cells that are genetically capable of reacting with all potential immunogens are present in the immune system. Generation of an immune response thus results from amplification and proliferation of that clone of cells best suited for a particular invading foreign antigen. Burnet also proposed that tolerance may occur when foetal immunocytes are exposed to and recognize a specific antigen - a process that leads to deletion of those clones of immunocytes. Support for the clonal deletion theory came from experiments conducted by **Billingham and Medawar**. They infused lymphoid cells from one inbred strain of mice (Strain A) into neonatal mice belonging to another inbred strain (strain B). Normally skin grafts from strain A mice would be rejected by strain B mice. However in this experiment, neonatal strain B mice, having been exposed to lymphoid cells from strain A mice became tolerant to cell surface antigens of the strain A mice. Thereafter strain B mice could receive skin grafts from strain A mice and tolerate the graft.

Unfortunately early tests to prove the clonal deletion theory were unsettlingly negative. Clinical immunologists began finding antibodies against normal body proteins such as insulin, in the blood of healthy people. These antibodies ought not to have been there if self reactive clones of B cells are deleted during foetal development. Obviously some self reactive clones of B cells were surviving. Solid proof that clonal deletions actually occurred, appeared in 1988 — 33 years after Burnet first proposed the idea. In independent studies, **Phillipa Marrack** and other workers demonstrated that certain self reactive clones of maturing T cells were deleted from the thymus and never reached the rest of the body. It is now apparent that T cells entering the thymus are schooled by two selection processes. In the first process, also called **positive selection**, T cells are taught to recognize the MHC proteins; an essential learning step for adequate T cell function (see Chapter 3). The second step, also called **negative selection**, is critical for self tolerance. Developing T cells are exposed to a pot-pourri of self antigens. Young T cells that take the bait and bind to self antigens die. Dangerously self reactive T cells are therefore weeded out in the thymus.

Matzinger has proposed that during neonatal life, whether encounter with an antigen results in tolerance or an immune response is determined by the prevailing host environment producing nonspecific cues 'sensing' danger. Neonatal T cells, she claims, are not intrinsically tolerisable but the systemic neonatal environment does predispose to tolerance. She has further suggested that the controlled death process of apoptosis is critical in preventing autoimmunity when old or surplus cells are disposed of.

Peripheral Tolerance

Clonal deletion, however, is not the entire answer. Some investigators believe, that it is incredible that every potential self antigen in the body must some how manifest itself in the thymus so that T cells can be screened. **Clonal anergy** has therefore emerged as a possible alternative mechanism for self tolerance. The term **clonal anergy** was coined by **Gustav Nossal** in the mid - 1970s. He found that under certain circumstances, B cells in culture could not be stimulated by antigen. The unresponsive B cells did not die but persisted in a lazy or anergized state. Although these experiments were elegant and informative, they do not reveal what ultimately happens to anergic, self reactive B cells especially in vivo. Almost 20 years ago **Peter Bretscher** and **Mel Cohn** proposed the **two signal hypothesis** for lymphocyte activation

and tolerance induction. They suggested that a B cell must receive two consecutive signals in order to be activated: signal 1 from the antigen and signal 2 from a second cell specific for the same antigen, for example, a T helper cell. If a B cell was, however, reacting to a self antigen, it would find itself alone and unable to receive signal 2 from a T helper cell, except in those exceedingly rare circumstances when a T helper cell is also reacting specifically to the same self antigen. Thus self reactive B cells receiving only signal 1 from the auto antigen could not be activated to respond without adequate T cell help. Such a B cell was therefore anergized (Fig 16.3).

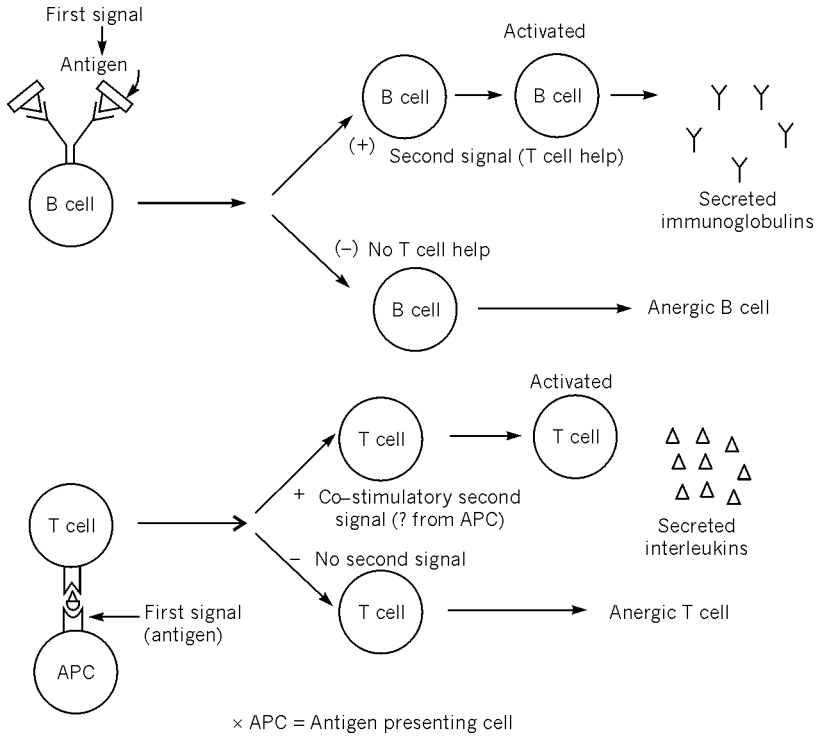

Figure 16.3. The two signal explanation for immunologic tolerance.

Although this model was originally proposed to explain B cell tolerance, **Schwartz and Jenkins** have now shown that a similar two signal theory can be used to explain T cell activation and tolerance (Figure 16.3). The essence of the two signal model is that simply binding of the T cell receptor to an antigen molecule is not enough to trigger T cell activation. The T cell must receive an additional signal of some kind from the antigen presenting cell. Without this second signal, a T cell becomes anergized and stays that way unless "reset" by the antigen presenters. It is thought that only a few types of specialized cells - macrophages for example, may be able to send this second signal needed to give T cells a push into its active, proliferative state. To date it is not known what precise steps are involved in the working of the clonal anergy theory.

Another mechanism of tolerance induction is **ligand-induced activation** or **antigen blockade**. In this mechanism, antigen, particularly multivalent antigen, interacts with antigen receptors on B cells, under circumstances that interfere with the processes of patching and endocytosis which are necessary for clonal expansion. Certain monovalent antigens may block

B cell receptors without leading to patch formation and certain multivalent antigens and immune complexes may immobilize membrane receptors and prevent patch formation (Figure 16.4). Either of these interactions renders lymphocytes unreactive to subsequent antigen exposure. In addition, occupancy of B cell receptors by non immunogenic forms of antigen, could prevent interaction with immunogenic forms such as antigen presented by macrophages. Antigen blockade produces reversible anergy because it does not lead to cell death.

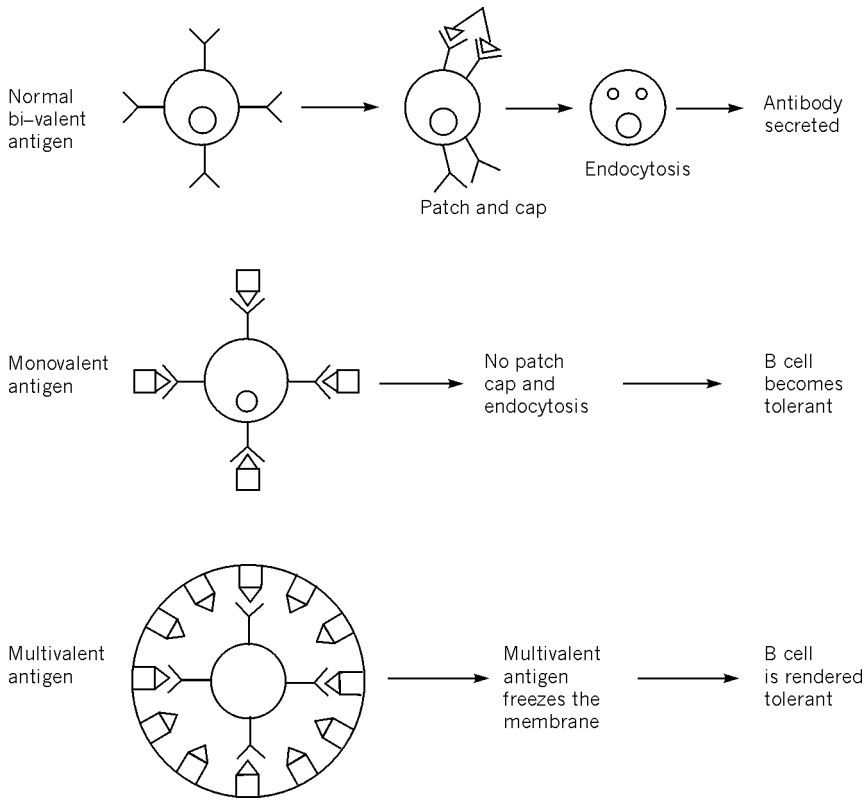

Figure 16.4. Antigenic blockade by monovalent and multivalent antigens.

Immune tolerance may also be induced by other mechanisms. Some antigens are sequestered from the immune system in locations which are not freely exposed to surveillance. These are termed immunologically privileged sites. Examples of such sites are the eye, CNS and testis.

Though the once popular suppressor T cell theory for tolerance seems to have fallen into disfavour, some researchers believe that the "data and the phenomena are still there"; however, there seems to be insufficient experimental evidence to prove the suppressor T cell phenomenon. It is the existence of a unique suppressor cell that is most controversial. The history of this fast changing field only shows that one year's unfashionable concept can become the next year's dogma.

At the molecular level, studies now indicate that binding of antigen to MHC gene products may play a role in immunologic tolerance. MHC Class II gene products on the surface of antigen presenting cells, have been shown to bind to processed protein antigens. It has been suggested

that self antigens may be differentiated from non self antigens by the way they bind to MHC Class II gene products, after they have been processed within the antigen presenting cells.

Autoimmunity

Autoimmune disease is defined as disease caused by immunologic reaction to self antigens. Such diseases are classified either as **organ specific** or **systemic** based on the primary location of the injury (Table 16.1).

Table 16.1: Classification of Autoimmune diseases

Disease	*Target of Antibody*
Organ specific diseases	
Myasthenia gravis	Acetylcholine receptors
Graves' disease	Thyroid stimulating hormone receptor
Thyroiditis	Thyroid
Insulin dependant diabetes with acanthosis nigricans	Insulin receptor
Insulin dependant diabetes with ataxia telangiestasia	Insulin receptor
Allergic rhinitis, asthma auto immune abnormalities	β 2-adrenergic receptor
Juvenile insulin dependant diabetes	Pancreatic islet cells, insulin
Pernicious anaemia	Gastric parietal cells, Vitamin B12 binding site for intrinsic factor
Addison's disease	Adrenal cells
Idiopathic hypoparathyroidism	Parathyroid cells
Spontaneous infertility	Sperm
Premature ovarian failure	Interstitial cells, corpus luteum cells
Pemphigus	Intercellular substance of skin and mucosa
Bullous pemphigoid	Basement membrane zone of skin and mucosa
Primary biliary cirrhosis	Mitochondria
Autoimmune haemolytic anaemia	Erythrocytes
Idiopathic thrombocytopenic purpura	Platelets
Idiopathic neutopenia	Neutrophils
Vitiligo	Melanocytes
Osteosclerosis and Meniere's disease	Type II collagen
Chronic active hepatitis	Nuclei of hapatocytes
Systemic diseases	
Goodpasture's syndrome	Basement membranes
Rheumatoid arthritis	Gamma globulin, Epstein-Barr virus-related antigens, types II and III collagen

SjÖgren's syndrome	Gamma globulin
Systemic lupus erythematosus	Nuclei, double-stranded DNA, single-stranded DNA, ribonucleoprotein, lymphocytes, erythrocytes, neurons, gamma globulin.
Scleroderma	Nuclei, centromere
Polymyositis	Nuclei, histadyl-tRNA synthetase, threonyl-tRNA synthetase
Rheumatic fever	Myocardium, heart valves, choroid plexus.

Mechanisms of Tissue Injury in Autoimmune Diseases

Three mechanisms are principally responsible for inflammation and tissue injury in autoimmune disease

- Cell lysis and release of inflammatory mediators triggered by auto antibodies
- Immune complex disease
- T-cell mediated damage

In the first mechanism, circulating antibodies react with modified or unmodified antigens on cell surfaces. The bound antibodies then stimulate the release of mediators of inflammation, trigger the complement pathway or activate K cells, via antibody dependant cellular cytotoxicity (ADCC). The latter two processes result in cell lysis.

In the second mechanism, complexes between auto antibodies and antigens form in the circulation or in inter cellular fluids. These immune complexes deposit in various tissues, including glomeruli, joints and blood vessels; they subsequently fix complement and cause inflammation and tissue injury. The site of deposition is determined by the physical properties of the immune complex, such as its size and charge.

In the third mechanism, sensitized T cells either injure cells directly or release lymphokines that amplify the inflammatory response. Although tissue injury caused by cell-mediated mechanisms may be important in autoimmune disease, its role is currently unclear.

Cell lysis by autoantibodies

Many autoimmune diseases are primarily initiated by the interaction between auto antibodies and cell surface antigens. One result of auto antibody binding is cell destruction. This occurs in Addison's disease, due to anti adrenal cell antibodies; in Hashimoto's thyroiditis, due to anti thyroid antibodies; in several other endocrinopathies and in some haemolytic anaemias and leukopenias.

Alternatively, auto antibodies may bind to cell surface receptors and interfere with their function. In myasthenia gravis, auto antibodies bind to acetylcholine receptors on the motor end plate. This leads to destruction of the receptor, defective neuro muscular transmission and resultant skeletal muscle weakness. Antibodies to many hormone receptors may act as either antagonists or agonists. Antibodies to the insulin receptor, in patients with diabetes mellitus, may antagonize insulin action by reducing the availability of the receptor. In Grave's disease, the long acting thyroid stimulator (LATS) is an auto antibody to thyrotropin receptor; it acts as an agonist, causing excessive secretion of thyroid hormones.

Immune complex deposition

In a number of systemic autoimmune diseases, tissue injury is mediated both by direct auto antibody binding and by immune complex deposition. In systemic lupus erythematosus (SLE), the prototype systemic autoimmune disease, antibodies bind to the erythrocyte, leukocyte, and platelet antigens, causing haemolytic anaemia, leukopenia and thrombocytopenia; anti-neuronal antibodies contribute to neurologic disease. Additionally, circulating immune complexes consisting of DNA- anti DNA are deposited in the glomerulus of the kidney and in other organs causing tissue injury. Two significant facts point to the role of immune complexes in SLE. First, patients demonstrate significant depletion of complement (C3) and neutrophils as a result of activation by complexes. Second, complement deficiencies which impair immune complex clearance (C1,C2 or C4,) are very strong predisposing factors for SLE.

In Rheumatoid arthritis, the auto antibody called rheumatoid factor, is usually an IgM, which is directed towards the Fc portion of the patient's own IgG. Complexes of rheumatoid factor IgM coupled with IgG, get deposited at various sites leading to the characteristic synovitis and vasculitis of rheumatoid arthritis. For a more complete description of the pathogenesis, diagnosis and treatment of the various autoimmune diseases, the reader is directed to refer to the many excellent textbooks of internal medicine available.

T-cell mediated damage

This term implies that the recognition of autoantigen by T cells leads to tissue destruction without requiring the production of autoantibody. There are a number of ways this can come about:

- Direct T cell cytotoxicity via $CD8^+$ CTL
- Self-destruction of tissue cells induced by cytokines, e.g. TNF α
- Recruitment and activation of macrophages leading to bystander tissue destruction
- Induction of target tissue apoptosis by the T cell membrane protein **FasL**

In most cases we do not know what the relative contribution of these factors is.

The Aetiopathogenesis of Autoimmune Disease

How does a finely balanced, perfectly tuned network of immune responses to foreign antigens break down to cause an immunological reaction that is self destructive? Many theories and mechanisms have been proposed for the generation of auto immune responses.

Exposure of sequestered antigens

If an antigen is sequestered within an organ during foetal development, then as per the theories of clonal deletion the immune system does not acquire tolerance to this antigen. No autoimmune response develops as long as these antigens remain unexposed. Should tissue damage or injury occur to expose these antigens, auto antibodies to these sequestered antigens rapidly appear in the circulation. Such an auto antibody reaction has been repeatedly demonstrated: auto antibody against sperm after vasectomy, against lens protein after eye injury, against heart muscle antigens after a myocardial infarct. These antibody responses are transient in most cases, apparently brief exposure is insufficient for inducing autoimmune disease, which may require persistent exposure to sequestered antigen.

Altered self antigens and molecular mimicry

Alteration of an antigen to which a host is tolerant, may allow immune reactions to the tolerant antigen to develop. Experimentally it has been shown that rabbits made tolerant to bovine serum albumin (BSA) can be stimulated to produce antibodies to native, unaltered BSA when they are immunized with chemically altered BSA. Alteration of self antigens occurs in the human system as a result of **viral** or **bacterial infections** or therapy with certain **drugs**. Antibodies to the I blood group are formed following *Mycoplasma pneumoniae* infection. Autoimmune haemolytic anaemia is associated with administration of α methyl dopa. The drug is thought to modify the red cell surface in such a way that certain B cell clones react against these modified red cells, causing haemolysis. Similarly, procainamide and hydralazine seem to provoke the production of antinuclear antibodies.

A related mechanism termed **molecular mimicry** comes into play when foreign antigens resemble self antigens. The encephalitis that sometimes results from the administration of the neural rabies vaccines is thought to occur due to an autoimmune reaction to human brain tissue, which cross reacts with neural tissue found in the neural vaccine. Similarly, in rheumatic fever, streptococcal antigens induce antibodies which react with components of the myocardium. Presumably streptococcal antigens resemble heart antigens enough, to elicit an autoimmune reaction.

The idiotype anti-idiotype network

The concept of anti idiotypic antibodies being stereochemically similar to the antigenic determinant, since both bind to the one antibody, has been discussed earlier in this chapter. Anti idiotypic antibodies that structurally resemble a viral antigen, will be able to bind to the receptor for the virus on the cell surface. This can stimulate a cytotoxic reaction against the receptor bearing cells. Therefore anti viral antibodies may induce anti idiotype antibodies that cross react with normal host tissues.There are several examples of autoimmune reactions that might be accounted for by the presence of cross reactive anti idiotype antibodies. Immunization of rabbits with antibodies to human thyrotropin produces anti idiotype antibodies that mimic thyrotropin reaction, by combining with thyrotropin receptors. This anti idiotype antibody resembles LATS found in patients with Grave's disease (Figure 16.5).

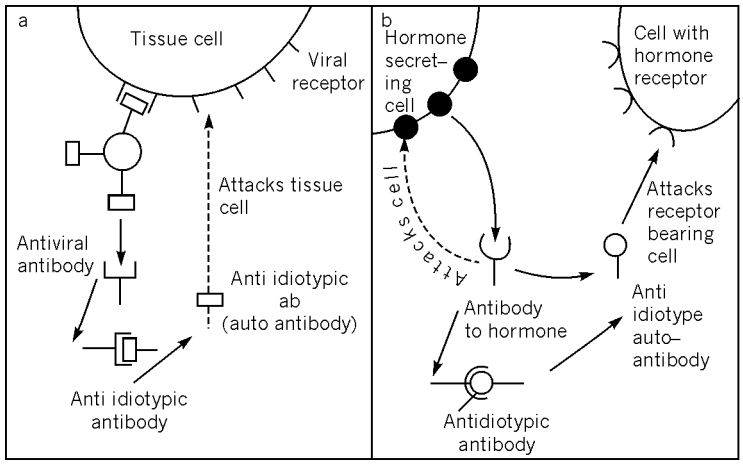

Figure 16.5. Idiotypic mechanisms leading to autoimmunity.

Abnormal expression of MHC antigens

Activated helper T cells are necessary for producing antibodies to most antigens, including self antigens. These cells become activated only when presented with antigen by cells that express MHC Class II antigens. Many self–reactive CD4+ T cells are eliminated in the thymus during maturation (see clonal deletion). However, several antigens such as neural and endocrine antigens are not present in the thymus, therefore those T cells that are self reactive with regard to such antigens cannot be screened and eliminated within the thymus. Such T cells escape into the peripheral tissues and are able to recognize the above self antigens when presented in association with MHC Class II antigens.

This potential self reactivity is usually prevented by the limited distribution of MHC Class II antigens, which are restricted to just a few cell types: the macrophages, T and B cells. These antigens can, however, be induced on other cells by viral infection, through the effects of interferons (see Chapter 12). During an inflammatory response an immunostimulatory environment is created by the release of cytokines which recruit and activate professional antigen presenting cells and provide support for T cell activation rather than anergy. As a result autoreactive T cells which were anergic may become activated. This concept is central to Matzinger's **Danger Hypothesis**. These theories therefore provide a possible mechanism for the induction of autoimmune disease by infection or inflammation.

Genetic and other factors

Genetic factors are known to play an important part in auto immune disease. Autoimmune diseases tend to occur in clusters in certain families. Hashimoto's thyroiditis and SLE occur more frequently among parents, children and siblings. Furthermore, there are strong associations between several autoimmune diseases and particular HLA specificities for example, HLA DR3 in Addison's disease and HLA DR4 in rheumatoid arthritis. However, no single genetic factor can account for any autoimmune disease. Although certain HLA - DR antigens are associated with SLE, most individuals bearing these antigens are healthy. Susceptibility must therefore be determined by more than one genetic factor or by a combination of factors.

Interestingly, certain inbred strains of animals spontaneously develop autoimmune disease. There is an obese line of chicken that spontaneously develop autoimmune thyroiditis, and the New Zealand Black (NZB) mouse develops autoimmune haemolytic anaemia. The hybrid of NZB with NZW (white) develops LE cells, antinuclear antibodies and fatal SLE. These animal models provide valuable insight into the study of autoimmune diseases.

Certain **hormonal factors** play a role in SLE and other autoimmune diseases. Most patients with SLE are women in their child bearing years. In addition, patients with SLE excrete excessive amounts of 16 α metabolites of estrones which have high oestrogenic activity. In certain strains of mice in which SLE develops, the autoimmune disease is aggravated by oestrogens and retarded by androgens.

Reviewing all the existing evidence regarding autoimmunity, it is clear that spontaneous autoimmune disease is a multifactorial phenomenon with immunologic, genetic, virologic, hormonal and other factors acting singly or synchronously to produce disease.

CHAPTER – 17

IMMUNOPOTENTIATION AND IMMUNOSUPPRESSION

An understanding of the normal regulatory mechanisms of the immune system has yielded an insight into the many ways the immune reaction may be enhanced or inhibited, should the need arise. Clinically, immunopotentiation has been effective in combating infectious disease, in the treatment of immunodeficiency states and in the search for a cure for cancer.

Immunosuppression has found relevance since the birth of organ transplantation, and in the treatment of autoimmunity and allergy.

Immunopotentiation

Vaccination

Vaccination has been used for over two centuries as a means of exploiting the immune system to protect the host against infectious agents. An understanding of antigen specificity and memory has been used to boost the immune response to infectious agents by artificially exposing the host to small amounts of the inactivated infectious agent. Immunization has thus offered protection against a number of potentially lethal infectious diseases such as small pox, poliomyelitis, pertussis, diphtheria, tetanus, mumps and rubella to name just a few.

Adjuvants

Responses to many killed vaccines need to be enhanced by substances that are collectively known as adjuvants. An adjuvant, by definition, is a substance when incorporated into or injected simultaneously with antigen potentiates the immune response.

Mode of action of adjuvants

(*i*) On antigen characteristics: Some adjuvants affect the way in which antigen is presented. The immune response is increased when protein antigens are precipitated by alum. Other adjuvants have a “depot” effect, in that they prevent rapid dispersal of antigen from the local tissues draining the injection site. This reservoir of antigen is then available either at an extra cellular location or within macrophages. The most common adjuvant of this type is Freund’s incomplete adjuvant, where antigen, in the aqueous phase, is emulsified with paraffin oil. Since paraffin oil can produce severe local reactions, it is not recommended for human use. Oils such as squalene and peanut oil seem to be better tolerated when compared to Freund’s incomplete adjuvant. Recent interest has been focussed on the use of liposomes, which are membrane bound lipid vesicles, as agents for presentation of antigen to the immune system. Liposomes seem to behave as storage vacuoles for the antigen, they also enter the macrophage and are presented in a more immunogenic manner to T cells.

(*ii*) On host immune response: Most adjuvants, however, do not affect the antigenic characteristics but act on the host immune response. Virtually all adjuvants stimulate macrophages, a good example is **Freund's complete adjuvant** which is made from incomplete Freund's adjuvant with the addition of killed mycobacterium or more recently, water soluble muramyldipeptide. Such adjuvants act directly on the macrophage or via the T cell. Besides improving antigen presentation, they enhance the accessory signals required for lymphocyte activation and proliferation.

Types of Adjuvants

(*i*) Organic adjuvants: Organic adjuvants include a variety of organic molecules obtained from bacteria. Muramyldipeptide (MDP) is a bacterial peptidoglycan. MDP increases both humoral and cellular immunity. It stimulates macrophages and may, as a consequence, recruit T cell help in vivo.

(*ii*) Synthetic adjuvants: Synthetic adjuvants that increase host immunity include levamisole and isoprinosine. Levamisole was initially introduced as an anthelminthic agent. It potentiates humoral and cellular immunity in a fashion that is T cell dependant. It has been used with some success in the treatment of cancer and rheumatoid arthritis. Unfortunately, these advantages are not without the risk of side effects of which agranulocytosis is the most serious.

Isoprinosine is a complex containing inosine, a purine precursor. In vitro isoprinosine promotes T cell mitogenesis. Its usefulness in the treatment of cancer has not been established.

(*iii*) Tuftsin : Tuftsin is a unique adjuvant that occurs naturally and has been synthesized as well. It is a four amino acid peptide-threonine-lysine-proline-arginine, homologous to a sequence in the constant region of the immunoglobulin heavy chain. Tuftsin primarily stimulates macrophages. Since it occurs naturally it probably has a physiologic role in host defence.

Lymphokines

The interleukins and interferons have been discussed extensively in Chapter 13. By recombinant DNA technology it is now possible to harvest unlimited quantities of IL-1, IL-2 and IFN γ, IFN β, and IFN α.

IFN α was the first to be produced in a large scale manner. IFN α has proven adjuvant properties, as assessed by reduction in tumour size, especially in lymphomas. IFN α appears to act both directly on tumour tissue and by activation of macrophages. IFNs α, β and γ are all used therapeutically in a number of clinical conditions (see Chapter 13).

Recombinant IL-2 was approved in 1992 for the treatment of metastatic and inoperable renal cell carcinoma. It has been used experimentally for malignant melanoma and HIV infection. It appears to restore both humoral and cellular immunity in nude (athymic) mice. IL-2 also appears to induce production of IFN α by T cells. Both the interleukins and the interferons are not free from side effects such as fever, malaise, myalgias, arthralgias and fluid retention.

An alternative approach to the in vivo use of IL-2 has been the induction of lymphokine activated killer (LAK) cells in vitro, by incubation of cells with IL-2. Under these conditions lymphocytes acquire cytotoxicity against a broad range of tumour cells. Cells activated in this manner have been used to cause tumour regression in mice. Clinical trials with these cells in humans have shown promising results.

Immunosuppression

Immunosuppression has been particularly useful is patients undergoing organ transplants and in the treatment of graft rejection, autoimmunity and allergy. Current treatment of graft rejection, autoimmunity or allergy is not antigen specific. The following are some of the agents used as immunosuppressives.

Cytotoxic agents

Cytotoxic agents such as cyclophosphamide, chlorambucil, azathioprine and methotrexate block cell replication and preferentially kill dividing cells. Cyclophosphamide and chlorambucil alkylate DNA in both dividing and resting cells, leading to cell death during the mitotic phase of cell division. Azathioprine and methotrexate block DNA synthesis, preferentially killing cells that are in the S (DNA-synthesis) phase of the cell cycle. The major use of these drugs has been to kill malignant cells. Cytotoxic agents, however, also suppress both humoral and cellular immunity. Because B cells or T cells that are stimulated by antigen, go through active proliferation-cytotoxic agents exert their killing action on these actively dividing cells. This may not be the only way that these drugs cause immunosuppression, none the less, they are an important part of the immunosuppressive therapy given during organ transplantation. They have also been used with some success in the treatment of various autoimmune diseases. These drugs, however, have numerous and serious side-effects.

Glucocorticoids

Glucocorticoids are potent immunosuppressive and anti inflammatory agents. They are used regularly in the treatment of graft rejection, allergies and asthma. The immunosuppressive effects of glucocorticoids are poorly understood, they appear to reduce the levels of circulating lymphocytes and monocytes and suppress the production of IL-1 and IL-2. Glucocorticoids have a diversity of actions and the clinical benefit seen in allergy and autoimmunity seems to be due to the various other actions of these drugs together with their immunosuppressive properties.

The chronic use of glucocorticoids is associated with serious side effects that hamper their continued use in some patients.

Cyclosporine and Tacrolimus

Cyclosporine is a cyclic polypeptide containing 11 amino acids, derived from soil fungi. Just after implantation of a foreign graft, foreign antigen sensitive T cells begin to get activated. Cyclosporine acts selectively on antigen-sensitive T cells in the G0 to G1 phase (see Chapter 12), and blocks the transcription of lymphokine mRNA, thereby suppressing IL-2 production. This effectively blocks T cell activation and proliferation. Resting T cells which carry memory for immunity to infectious agents are spared. It has also been shown that the drug leads to the development of active “suppressor” T cells, which could actively maintain tolerance to the grafted tissue.

Cyclosporine has been used successfully to prevent graft rejection and in the treatment of graft versus host disease. It has little toxicity for dividing cells in the gut and bone marrow. Side effects of cyclosporine include nephrotoxicity and hepatotoxicity. It is also associated with an increase in B cell lymphomas, though the incidence reported is relatively low.

Tacrolimus is a macrolide antibiotic that works in a mechanism similar to that of cyclosporine to prevent allograft rejection.

Antilymphocyte antibodies

Antilymphocyte globulins have been shown to deplete lymphocytes and produce immunosuppression. They prolong graft survival in recipients, though long term effects have been variable. Monoclonal antibodies to the T cell surface antigen, CD3 and the IL-2 receptor have been used for treating host rejection of allografts. Both agents have been associated with several problems including unpleasant side effects.

Monoclonal antibodies to other T cell surface antigens are being investigated in animal models, as potential immunosuppressive agents.

CHAPTER – 18

TRANSPLANTATION IMMUNOLOGY

The replacement of hopelessly diseased vital organs with healthy donor organs has offered hope of a better quality of life to many patients. The science of transplantation has moved from its once faltering position in the 1960s to that of a promising and life- enhancing endeavour.

Classification of Grafts

The major classification of grafts is based on the relationship between the donor and the recipient. There are three main types of grafts:

- a graft taken from one location in an individual and returned to the same individual at a different location is called an **autograft/ autologous graft**.
- an **allograft/ allogeneic graft** is taken from one individual of a given species and placed in another individual of the same species.
- a graft between two genetically identical individuals, as in identical twins is called an **isograft/ isogeneic graft**.
- a **xenograft** is taken from an individual of one species and placed in an individual of a different species.

Grafted cells, tissues or organs may be placed in their normal location as in an **orthotopic** graft. Grafts may be placed in an atypical location, when they called **heterotopic** grafts. Almost all clinical interest is centered around allografts and the immunology of transplantation concerns itself mainly with the survival of the allograft.

Immunology of Graft Rejection

First and second set reactions

When a graft is undergoing rejection for the first time, there is very rapid invasion by polymorphs and lymphoid cells, including plasma cells. Thrombosis and acute cell destruction can be seen in three to four days. This "**first time**" reaction to a foreign graft is known as the **first set reaction**.

Second set rejection occurs during subsequent grafting, taken from the original donor or a related subject. It does not occur if the allograft has been donated by an entirely unrelated subject with a completely different set of tissue antigens. (Such grafts are rejected as first set of reactions). The recipient of a second graft from a donor who has already been rejected, will mount an accelerated rejection of the second graft. Since T cells are implicated in graft rejection, it demonstrated that they are primed and retain memory of the first contact with graft antigens.

During rejection humoral antibodies, with specificity for the graft antigens are produced. Such antibodies are produced in a T cell dependant manner i.e., with T cell help.

Immune Mechanisms in Graft Rejection

Immune mechanisms in graft rejection are best illustrated using rejection of the grafted kidney as a model.

A. Acute rejection

Antigen presenting cells (APC) in the grafted tissue provide the primary stimulus. These MHC Class II-positive cells are mainly dendritic cells and to a lesser extent - monocytes. These cells are necessary to present antigen in a form that recipient lymphocytes can recognize. These cells are sometimes called “passenger cells” since they come along with the graft. If they were not so readily available, foreign tissue antigen would have to be processed and presented by recipient APCs. As shown in Figure 18.1 a, the antigen presenting cells bearing MHC Class II antigens are recognized by CD4+ T cells or helper T cells. Together with IL-1 which is secreted by the “passenger APC”, the CD4+ T cells get activated and proliferate, constituting an in vitro mixed lymphocyte reaction (MLR). Once activated, these cells release IL-2, which is an essential factor in the activation of CD8+ T cells (cytotoxic T cells) and B cells. Clonal proliferation of cytotoxic T cells and B cells results.

The precise mechanism by which cytotoxic T cells kill graft cells is unclear. Direct cytotoxicity is a possibility as cytotoxic T cells recognize MHC Class I antigens, which are displayed on virtually all human cells. Another important consequence of T cell activation is the release of IFN. IFN induces increased expression of HLA-A,HLA-B and HLA-DR antigens on graft tissue, which makes the graft more vulnerable to T cytotoxic mediated killing. It also activates monocytes to mediate a destructive delayed hypersensitivity response against the graft (Figure 18.1 b). In addition, activated CD4+ cells release IL-4, IL-5 and IL-6,which cause B cells to produce antibody. Antibody mediated damage takes place via complement activation or by recruitment of antibody dependant cell mediated cytotoxic effector cells (Figure 18.1 b).

This type of acute rejection occurs within 10 days of transplantation and is characterized by dense cellular infiltration. High dose corticosteroids are often used to treat acute rejection. Corticosteroids function through several mechanisms (see Chapter 17). They are especially useful in treating graft rejection because they reduce the capacity of APCs to express Class II antigens and to release IL-1. They inhibit T cell activation in the recipient and block release of IL-2. They are also known to produce lymphocytopenia especially of CD4+ cells. If the response to corticosteroids is inadequate, antilymphocyte antibodies may be necessary. Monoclonal antibodies to CD3 T cells and those against the IL-2 receptor are used.

B. Hyperacute rejection

Hyper acute rejection occurs within minutes of transplantation and is characterized by sludging of red cells and microthrombi in the glomerulus. This type of rejection occurs in individuals with pre existing antibodies to blood group antigens or in those that are pre sensitized to MHC Class I antigens of the donor, through blood transfusions. Antigen antibody complexes formed during rejection, fix complement and complement activation ensues, followed by activation of the clotting pathway. This leads to microthrombi within glomerular capillaries, leading to severe ischaemia and necrosis of the graft. To date, there are no effective means to treat this condition, except by ensuring that pre sensitization has not taken place.

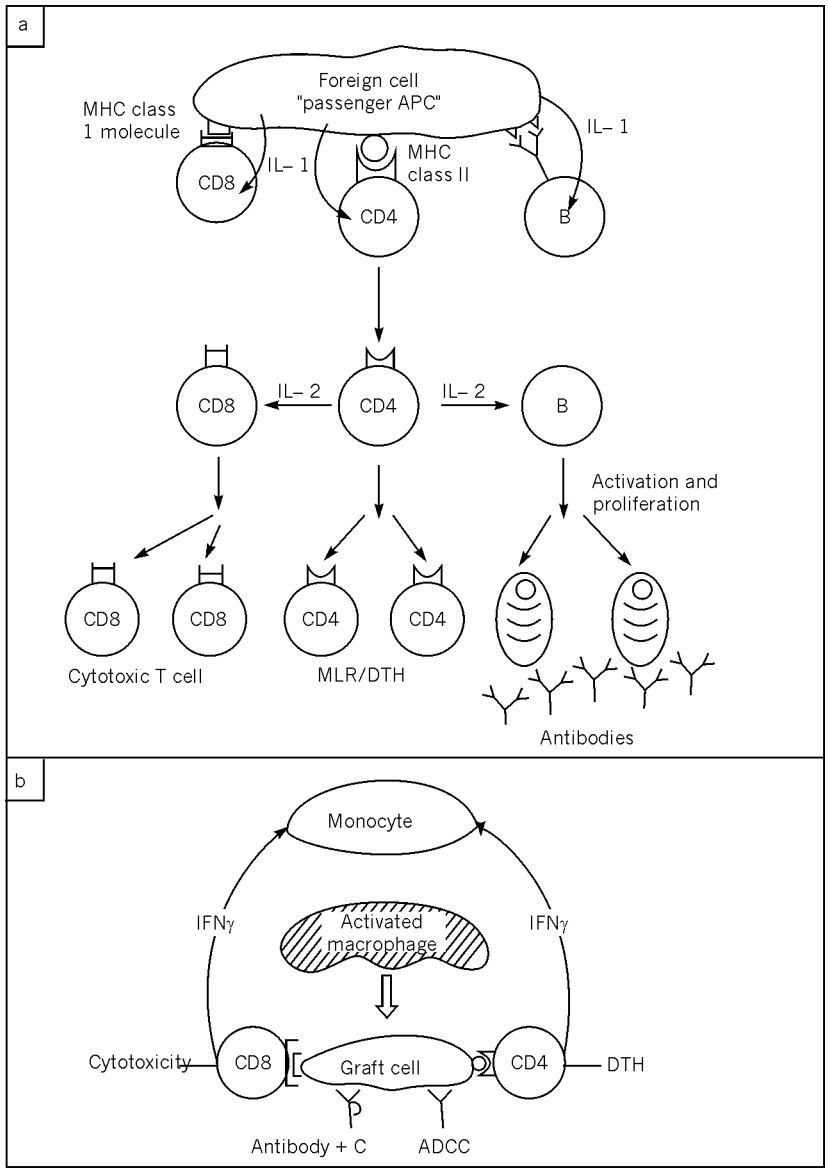

Figure 18.1. (a) Cellular responses to grafted antigens
(b) Mechanisms of allograft rejection
APC = antigen presenting cell
DTH = delayed type hypersensitivity
MLR = mixed lymphocyte reaction
ADCC = antibody dependant cellular cytotoxicity

C. Chronic rejection

Chronic rejection occurs months to years after transplantation. It is characterized by a narrowing of the vascular arterial lumen, owing to growth of endothelial cells that line the vascular bed. The actual mechanism involved in this overgrowth of endothelial cells is unknown. Monocyte release of IL-1 and platelet and endothelial cell release of platelet derived growth factors are the two factors implicated. Initially the proliferating endothelial cell lesion is reversible, but once it progresses to fibrotic changes, it is unresponsive to treatment and progresses to graft ischaemia, interstitial fibrosis and loss of renal function.

Precautions against graft rejection

The typing and cross matching of the **ABO blood groups** is an essential test performed on all recipients and potential donors. The ABO system is present not only on red blood cells, but also on the vascular endothelium of the graft. Hence renal transplants are performed only between ABO - compatible pairs. Very rapid graft rejection occurs if there is ABO mismatching between donor and recipient.

Ideally all recipients and potential donors need to have a complete tissue typing profile done. The HLA-A, B, C and D/DR tissue antigens are typed (see Chapter 10). The use of drugs such as cyclosporine A, have greatly diminished the effects of HLA mismatching. However, most transplant specialists will insist on a favourable degree of matching at the DR locus. Statistics show that matching at the DR locus is of greater benefit than the B loci which in turn is more advantageous to graft survival than the A loci.

Another test that is commonly done is the **mixed lymphocyte culture** test. This test evaluates the recipient's in vitro proliferative response to mismatched D/DR antigens on the donor's cells, when donor and recipient cells (monocytes from blood) are mixed. A mixed lymphocyte reaction (MLR) occurs if there is mismatching at the D/DR locus. However, if only one haplotype is mismatched, a weak MLR results and chances of graft survival are excellent (90%). If a strong MLR occurs, even within family members, graft survival rates fall to 60%. Some centers do not recommend transplantation in such cases.

A **cross match test** is used to determine the presence of any preformed antibodies (presensitization) to donor HLA antigens. The test is done using the patient's most recent serum and the donor's lymphocytes (from peripheral blood). If the donor's cells are killed by the patient's serum, it indicates a positive cross match and therefore the presence of preformed antibodies to donor cells. Positive cross matches are usually a contra indication to transplantation.

Tissue and Organ Transplantation

Corneal grafts

Corneal grafts constitute some of the most successful grafts done to date. This is because they are avascular and do not sensitize the patient. They survive without immunosuppressive therapy. Grafts of cartilage are successful in the same way and remain protected by the matrix. Such tissues are referred to as **immunologically privileged sites**; due to their poor accessibility with regard to the immune system.

Kidney transplants

The first successful renal transplant was performed at Peter Bent Brigham Hospital in 1954. Since then, thousands of successful kidney transplants have been carried out and survival

rates are high. Advances in tissue typing and improved understanding of immunosuppressive therapy, especially cyclosporine, has contributed to the success of renal transplants. It has now been firmly established that multiple blood transfusions prior to grafting, aids survival of the graft. The reason for this is not known, it may be due to the presence of blocking antibodies or to the generation of anti idiotypic suppressor T cells, as a result of the transfusion.

Heart transplants

The first successful human heart allograft was performed in 1967. Cardiac transplants are indicated in patients aged 40 or below with end stage coronary artery disease, cardiomyopathy, rheumatic heart disease or congenital heart disease. Absolute contra indications are severe pulmonary hypertension, infection and cancer. The one year survival rate for heart transplants stands at around 80%, mainly due to the introduction of cyclosporine. Full HLA matching, though ideal, is not possible. Single DR haplotype compatibility gives 90% survival after 3 years, this falls to 65% when both haplotypes are mismatched.

Liver transplantation

The most common indication for adult liver transplantation has been hepatitis B antigen negative, post necrotic cirrhosis or chronic active hepatitis. Infants and children with congenital or developmental anomalies of the bile ducts, benefit from successful liver transplants. Other indications for liver replacement in children are inborn errors of metabolism such as Wilson's disease and tyrosinaemia. The survival rate for one year is 68% for adults and 75% for children. Common complications after liver transplants are organ ischaemia, infection and rejection.

Bone-marrow transplantation

Over the past two decades, **allogeneic bone marrow transplantation** (BMT) has evolved from an experimental procedure reserved for patients with refractory leukemia into a rapidly expanding area of clinical investigation that offers potential cure for patients with aplastic anemia, acute and chronic leukemia, breast cancer, and selected types of lymphoma. The objective of BMT is to provide a healthy stem cell population that will differentiate into blood cells to replace deficient or pathologic cells of the host.

Patients with acute myeloid or lymphoblastic leukemia may benefit from BMT. Patients with acute myeloid leukemia transplanted in first remission can now expect an approximately 50 to 60% likelihood of long-term disease-free survival. Similar probabilities are also achievable after transplantation of adults with acute lymphoblastic leukemia in first remissions. Probability of relapse correlates with remission status at the time of the transplant, ranging from 20% in first remission to 60% with more advanced disease. Long-term survival for patients with chronic myelocytic leukemia who receive BMT in the phase of remission is 60 to 70%.

Pediatric BMT has expanded because of its potential for curing children with genetic diseases (e.g., thalassemia, sickle cell anemia, immunodeficiencies, inborn errors of metabolism).

Marrow can be procured from unrelated living donors. Related donors who are not HLA-identical have been used with increasing frequency. Results with either procedure suggest long-term disease-free survival probabilities of 30 to 50% in patients with acute and chronic leukemia or aplastic anemia; i.e., in most situations the results are somewhat inferior to those with marrow from HLA-identical siblings.

Another option for BMT is **autologous transplantation** (removal of a patient's own marrow when a complete remission has been induced, followed by ablative treatment of the

patient with the hope of destruction of any residual tumor and rescue with the patient's own bone marrow). Since an autograft is used, no immunosuppression is necessary other than the short-term high-dose chemotherapy used for tumor eradication and bone marrow ablation; posttransplant problems with GVHD are minimal. Indications for autologous BMT are relapsed, chemotherapy-sensitive lymphoma, in which a 30 to 40% success rate has been achieved, and acute leukemia in remission, in which 20 to 50% success rates have been observed. Success rates are inferior with more advanced disease and with responsive solid cancers (e.g., breast or germ cell tumors). Two major obstacles remain for successful application of autologous BMT: the possibility of contamination of the marrow inoculum with tumor cells, and the absence of graft-vs-tumor activity (in contrast with that seen in allogeneic BMT), both of which contribute to the observed higher rates of tumor recurrence.

Peripheral blood stem cell transplants

A relatively recent development in stem cell transplantation is the use of peripheral blood cells instead of stem cells from bone marrow. Peripheral blood stem cells (PBSCs) are obtained from circulating blood rather than from bone marrow, but the amount of stem cells found in the peripheral blood is much smaller than the amount of stem cells found in the bone marrow. Peripheral blood stem cells can be used in either autologous or allogeneic transplants. The majority of PBSC transplants are autologous. However, recent clinical studies indicate that PBSCs are being used more frequently than bone marrow for allogeneic bone marrow transplantation.

The advantages of PBSC transplants when compared to bone marrow transplants are: in allogeneic transplantation, haematopoietic and immune recovery are faster with PBSCs which reduces the potential for disease recurrence, primarily graft-versus-host-disease. In autologous transplantation, the use of PBSCs can result in faster blood count recoveries. Also, some medical conditions exist in which the recipient cannot accept bone marrow stem cell transplants, but can accept PBSC transplants. Some possible disadvantages to PBSC transplant versus bone marrow transplantation are: so much more fluid volume is necessary to collect enough PBSCs that, at the time of infusing the new stem cells into the recipient, the fluid can collect in the lungs or cause temporary kidney problems. Also, the time commitment for the donor for a PBSC transplant is considerable. When the PBSCs are being collected, several outpatient sessions are needed and each session lasts approximately two-four hours.

Other organs

Transplantation of the pancreas and other endocrine organs and the lung are being intensively researched. The feasibility of such transplants will improve with advances in techniques, therapy and supportive care.

Graft Versus Host Disease (GVHD)

When immunocompetent donor cells are grafted into an immuno-compromised host, graft versus host disease can occur. The grafted cells survive in the immuno-compromised host even though they are histo-incompatible, as the recipient is unable to mount an immunological reaction against them. In time, these immunologically competent cells recognize host antigens and react immunologically against them. Therefore, instead of a host reacting against the graft; the reverse takes place where the graft mounts a reaction against an immuno-suppressed host, incapable of fighting back.

A major problem in allogeneic bone marrow transplantation (BMT) is the prevention and control of GVHD. Symptoms and signs of acute GVHD are fever; exfoliative dermatitis; hepatitis with hyperbilirubinemia; vomiting; diarrhoea and abdominal pain, which may progress to an ileus; and weight loss. Although increased knowledge of the major histocompatibility complex has aided understanding of the aetiology of GVHD, patients who are matched at the A, B, C, and DR loci still have a 30 to 60% incidence of GVHD. Although the introduction of cyclosporine in the early 1980s has greatly reduced both the incidence and severity of GVHD, it continues to be the major cause of mortality and severe morbidity after allogeneic BMT.

About a third to half of BMT recipients develop a more indolent, chronic form of GVHD. Although the skin, liver, and gut remain the organs primarily affected, other areas of involvement (e.g., joint, lung) are also noted. Interestingly, bronchiolitis obliterans similar to that seen after lung transplantation can occur. Ultimately, 20 to 40% of the patients die of complications associated with GVHD, the incidence being higher when donor marrow is not from an HLA-identical sibling. In patients without chronic sequelae of GVHD, all immunosuppression can be stopped 6 months after BMT, making late complications rare in these patients, in contrast with the continued need for immunosuppressants and resulting complications in solid organ transplant recipients.

One area of active clinical research aimed at reducing the incidence of GVHD has been the removal of T cells from the donor marrow with monoclonal antibodies or mechanical separation before reinfusion of the marrow. T-cell depletion has been very effective in decreasing both the incidence and severity of GVHD; however, the incidences of engraftment failure and relapse are increased. A possible explanation is that the cytokines generated in the graft-vs-host reaction promote stem cell multiplication and maturation necessary for engraftment. Patients who develop GVHD have significantly lower relapse rates, suggesting that T cells responsible for GVHD are probably involved in a graft-vs-leukemia effect. Other agents used to prevent or treat GVHD include methotrexate, corticosteroids, antithymocyte globulin, and monoclonal antibodies against antigens expressed on mature T cells.

GVHD may also follow blood transfusions in exceptional cases, since even small numbers of donor T cells can induce this reaction. Such situations include intrauterine foetal blood transfusions and transfusions in immuno-suppressed patients (e.g., BMT recipients, leukemia, lymphoma, neuroblastoma, Hodgkin's and non-Hodgkin's lymphoma). Blood products to be given to patients at risk should be irradiated to prevent development of GVHD.

The Enigma of the Foetal Graft

Pregnancy in mammals acts much as a graft does, bringing into direct contact two genetically distinct individuals in the foetus; one half of all genes come from the mother and the other half from the father. In the case of the foetus, however, there is no dramatic immune response by the mother against the graft and pregnancy is not compromised. How the foetus escapes rejection and flouts the laws of tissue transplantation remains unknown.

It has been hypothesised that the maternal immune system may be prevented from recognizing the foetal tissue as foreign and/or the cells of the maternal immune system may be prevented from mounting an immune response in a number of ways.

Foetal tissue displays a unique type of MHC class I molecule encoded by the gene HLA-G. This molecule is considered to be a non-classical MHC molecule because of its low allelic variation (only two polymorphs are known) and restricted tissue distribution (found only in the placenta) A study by Kovats *et al*, localized the expression of HLA-G to the trophoblast cells, the embryonic

contribution to the placenta. Its function in the placenta may be inhibitory as it may prevent the activation of decidual cytotoxic T cells and NK cells. (The decidua is the maternal contribution to the placenta). Studies have shown that cells expressing HLA-G are unable to activate T cells and inhibit cytotoxic lysis mediated by NK cells.

Another lymphoid cell seems to play an important role in a successful pregnancy: the macrophage. Classically activated macrophages mediate proinflammatory responses, which involve T_h1 cell activation and cytokine secretion. Cytokines such as IL-1, IL-6 and IL-12 promote inflammation. In contrast, "alternatively" activated macrophages promote anti-inflammatory responses by secreting cytokines, such as IL-10 and IL-1-receptor antagonist, that down regulate the inflammatory response and inhibit the activation of T_h1 lymphocytes. Alternatively, activated macrophages may create an immunosuppressive environment in the placenta.

Recent studies have shown that a complement inhibitor, may be another way that the maternal immune system is regulated to promote a successful pregnancy.

Another mechanism for preventing foetal rejection that has received some attention from researchers is tryptophan catabolism. Interestingly, it seems that tryptophan catabolism is employed by the immune system to accomplish another goal: to prevent rejection of the foetus. Tryptophan deprivation may reduce or inhibit the immune response in two ways: it may inhibit lymphocyte proliferation or it may halt the manufacture of effector proteins.

Subsequently, understanding these mechanisms may lead to treatments that will help women who experience recurrent spontaneous abortions maintain a pregnancy and carry a baby to term. Understanding these processes may also help us in preventing rejection of donor tissue grafts by the recipient, since the goals of a pregnancy and tissue grafts are relatively the same: to sustain a foreign tissue within an immunocompetent host. However, all experimental evidence so far appears equivocal and despite several decades of work the enigma of the foetal graft still remains.

CHAPTER – 19

TUMOUR IMMUNOLOGY

Immunology and cancer has had a long and sometimes frustrating association. The idea that immunologic processes could ward off malignant cells was reinforced in the 1950s when it was accepted that one of the important functions of cellular immunity was to execute surveillance against malignant cells. The concept of immune surveillance addresses one essential question. Do tumour cells show differences from their normal cellular counterparts that the immune system can recognize? If so, it would be interesting to know whether tumour associated antigens are specific for the particular cancer involved or whether they represent simple differentiation into malignant cells. If tumour associated antigens do exist, does the immune response directed against these antigens give rise to death of the tumour cell?

Surface Antigens on Tumour Cells

Tumour antigens fall into **2 broad categories:**

- **tumour specific antigens (TSA)** are unique, found only on tumour cells and are eminently positioned as targets for immunologic attack.
- **tumour-associated antigens (TAA)**, by contrast, are found an tumour cells and some normal cells as well.

Unique Tumour Specific Antigens

Unique tumour specific antigens are found only on tumour cells and not on other cells of the host. There are several examples of unique tumour specific antigens (TSAs)

(a) *Virally Induced Antigens*

The best example of unique TSAs are those induced by viruses. In mammals, several RNA virsuses (example: retroviruses) and DNA viruses (example: herpes viruses) cause malignant transformation. The best example and the greatest triumph so far in tumour immunology is that of **Marek's disease in chickens,** caused by a herpes virus. The virus infects the lymphocyte and transforms it; a unique protein is found on the membrane of the transformed lymphocyte when the viral DNA integrates with the host cell genome. All tumours due to this virus carry this surface antigen and antibodies to the protein are detectable in host sera. It is now possible to immunize animals prophylactically against Marek's disease, using either the whole virus or the TSA.

Tumour cells induced by **RNA viruses** express antigens coded for by the viral protein in addition to their own cell surface antigens. These may be viral envelope antigens or intra viral proteins. Antigens of virally induced tumours show extensive cross reactivity; immunization of animals with any of these viruses provides protection against a variety of similar viruses.

Common oncogenic RNA viruses are the human T cell leukaemia virus (HTLV) in man and the feline leukaemia virus (FeLV) in cats. In many of these oncogenic viruses, it has been shown that viral antigens activate cellular oncogenes leading to cell transformation. Some of these oncogene products will render malignant cells sufficiently disparate from normal cells. This could also explain the extensive cross reactivity between the TSAs of oncogenic viruses.

(*b*) *Chemically or Physically Induced Tumour Antigens*

Tumours induced by chemical carcinogens such as methyl cholanthrene (MCA) express unique and individually distinct tumour antigens. The antigen is specific to the tumour produced in that MCA induced tumours in one mouse will not share common antigens with an MCA induced tumour on another mouse, although they are of the same inbred strain. In fact multiple MCA induced tumours on the same mouse are all immunologically distinct from each other. The precise mechanism accounting for this phenomenon is not known. It may result from an altered expression of normal molecules on the cell due to gene mutations. Alterations in both major and minor histocompatibility antigens on tumours have been described in chemical carcinomas. The same mechanism is said to operate in tumours due to ultraviolet light or radiation. Generation of an enormous variety of distinct tumour antigens makes it unlikely that immunization against such cancers will ever be possible.

(c) Antigens of Spontaneous Tumours

Spontaneous tumours are those that have no known inducing agent. Though antigens differing from normal cellular antigens have been identified, it has not been possible to characterize a unique TSA from such tumours.

Tumour Associated Antigens

Though tumour associated antigens are, to a large extent, specific to the tumours that display them, some normal cells may also express such antigens at particular stages of differentiation. Monoclonal antibodies have now permitted the characterization of these antigens and are now widely used to diagnose some of these tumours.

(*a*) *Oncofoetal antigens*

Oncogenes were discovered in cancer-causing viruses. Most oncogenes were actually present in the host cell, where they functioned in regulated cell growth. The host cell gene was called a **proto-oncogene**. When transduced by the virus and expressed under the control of a viral promotor, the gene product contributes to the unregulated growth of the tumour cell. Since proteins encoded by proto-oncogenes are expressed by normal cells, their over-expression on tumour cells would qualify them as tumour-associated antigens.

Tumour cells can sometimes “switch on” genes which are associated with growth and development of the foetus. Consequently, antigens which are associated normally with embryogenesis and are not detectable in the adult begin to appear during tumour growth. The prototype oncofoetal antigen is **carcino embryonic antigen (CEA)**, which is an improperly glycosylated glycoprotein found on foetal gut and human colon cancer cells, but not on normal adult colonic cells. Such abnormal glycosylation may be the cause of the observation that certain human gastric carcinoma cells display ABO blood group antigens different from the host ABO blood group.

Elevated serum CEA also occurs in inflammatory conditions of the colon and pancreas and also in cancers of the breast and pancreas. Measurement of serum CEA is not useful for diagnosing cancer, it is useful in monitoring response to treatment.

Alpha fetoprotein is a secreted tumour antigen and is the foetal equivalent of albumin. It is found in the serum of patients with hepatomas and teratomas and can be used as a marker for the presence of such cancers. It is, however, not a suitable target for tumour rejection.

(*b*) *Differentiation antigens*

Differentiation antigens are unique to the histogenetic type of tumour, rather than to the process of cellular transformation. Thus, there are antigens common to melanomas or neuroblastomas and they reflect the stage of differentiation at which particular tumour cells are arrested. Therefore, tissue specific tumour antigens represent differentiation antigens of the stages of cell lineage from which tumours arise. Hence they will not be entirely specific to tumour cells and populations of normal cells of the same lineage will carry similar antigens. An immune response against such antigens carries the danger of attack against normal cells as well.

Immune Responses to Tumour Antigens

Where tumour antigens have been shown to exist, both T cell cytotoxicity and tumour specific antibodies have been demonstrated.

Cellular mechanisms in tumour immunity

(*a*) *T cells*

T cell responses are the most important means of control against antigen bearing tumour cells. Both T cell populations : the MHC Class II restricted T helper cells and the MHC Class I restricted T cytotoxic cells play a role in control of tumour growth.

Since most tumour cells express MHC Class I antigens, the T helper (CD4+T) cells cannot directly recognize these tumour cells. Therefore T helper cells are dependant upon antigen presenting cells (APC), such as macrophages, to present the tumour antigens in association with MHC Class II antigens. This leads to T helper cell activation and subsequent IL-2 secretion, which in turn activates the cytoxic T cell (CTL/CD8+T) cells, macrophages, NK cells and B cells. T helper cells also produce lymphotoxin and tumour necrosis factor, which may directly lyse tumour cells.

The activated CTLs proliferate in response to IL-2 provided by the T helper cell. They then recognize tumour targets in association with the ubiquitous MHC Class I antigen and cause direct cell mediated cytotoxicity.

(*b*) *Macrophages*

Macrophages are important not only as antigen presenters; evidence suggests that they may also act as potential effector cells mediating tumour lysis. Macrophages activated by macrophage-activating factor (MAF) become cytolytic. MAF is a lymphokine secreted by T cells following antigen stimulation. Since macrophage activation is not antigen specific, they possibly

play a role in lysing cells with variant tumour antigens, which have therefore lost their potential to activate T helper cells.

(*c*) *Natural killer (NK) cells*

NK cells have the ability to kill a wide range of tumour targets in vitro. The precise mechanism of this killing is unknown. Recognition and activation do not appear to be antigen specific. Cytolysis by NK cells is mediated by the release of one or more cytotoxic factors. The cytotoxic activity of NK cells is augmented by IL-2 and interferon, demonstrating that T cell activation enhances NK activity. NK cells represent the first line of host defence against tumour growth, at both primary and metastatic sites. It is also an effector mechanism that can be recruited by T cells to supplement their tumoricidal activity.

There are other cytotoxic cells that appear to kill tumour cells apart from the NK cells. These naturally cytotoxic cells are resistant to suppression by glucocorticoids. Lymphokine activated killer (LAK) cells can be induced by very high doses of IL-2. They are phenotypically different from NK cells and kill a much broader range of tumour targets. The physiologic role of these cell types in controlling tumour growth in vivo remains to be elucidated.

(*d*) *B cells and antibody dependant cellular cytotoxicity*

Ever since antibodies to putative tumour antigens have been detected in serum, a potential role for host defence in cancer has been suggested. However, the exact mechanism by which they operate remains unclear.

There are two major mechanisms by which antibodies may mediate arrest of tumour growth. Complement fixing antibodies fix to tumour antigens, recruit complement and cause tumour cell lysis. Another mechanism is via antibody dependant cellular cytotoxicity (ADCC), where an immunoglobulin (usually IgG), forms a bridge between the target tumour cell and an effector or K cell (usually macrophages or granulocytes). This brings the killer (K) cell into proximity with the tumour cell and cell lysis ensues. There is evidence to suggest that ADCC is the more important mechanism of the two described.

Mechanisms by which tumours escape immune surveillance

That the immune mechanism plays a role in the host-tumour relationship is evidenced by the finding that immunosuppressed persons are more susceptible than normal individuals to cancers. Leukaemias are a common occurrence in radiation victims. In immunosuppressed patients and in those with immunodeficiency diseases, there is an increased incidence of cancers. Cancers also seem to hit older individuals when the strength of the immune system begins to wane.

Several reasons have been suggested, from time to time, to explain tumour escape from the watchful eye of immune surveillance.

Poorly immunogenic variants

It is possible that variants with diminished expression of tumour antigen are generated as a means of escape from the immune mechanism. This phenomenon of “sneaking through” the immune surveillance, selects out only those cells which survive, to grow into unchecked tumour masses. Tumours may also escape by changing their surface antigens, leading to selection of those clones lacking the original surface antigen.

Rapid growth

If an immune response is to cause tumour regression, it must destroy cells more rapidly than the new cells that are generated by tumour growth. In some experimentally induced tumours, it has been shown that immune destruction is too slow to do more than just slow down tumour growth.

Blocking factors

It has been shown that incubation of tumour cells with host serum prevented T cell mediated killing of tumour cells by the presence of antibody coating the tumour cells. The precise nature of these factors that block anti tumour immune responses are not known. Investigators have found non cytolytic antibody, immune complexes and free tumour antigens to act as potential blocking agents. Antibodies and immune complexes mask target antigens and prevent T cell recognition and free tumour antigens mop up cytolytic antibody and T cell recognition sites, allowing in situ tumour masses to grow unchecked.

Suppressor mechanisms

The tumour itself may suppress the immune response. Deficient cellular immunity can be associated with recurrence and dissemination of tumours, although cause and effect are difficult to distinguish. This deficiency has been repeatedly shown in various tumours, most dramatically in Hodgkin's disease, which appears to involve a variable defect in T-cell function. Decreased IL-2 production, an increase in circulating soluble IL-2 receptors, and induced defects in antigen-presenting cell function may also be involved. Defective function of the T cells infiltrating the tumour has been shown and can be overcome by sufficient antigen presentation by antigen-presenting cells and appropriate cytokine support. Deficient humoral immunity is commonly associated with neoplasms involving abnormal B-cell derivatives (e.g., multiple myeloma, chronic lymphocytic leukemia).

It is possible that tumours produce immunosuppressive agents that suppress the immune response in vivo. Alpha fetoprotein is a tumour product known to have immuno suppressive activity. Prostaglandins released by macrophages of tumour bearing hosts have demonstrable immunosuppressive activity.

Alteration in the expression of MHC coded antigens

The expression of MHC Class I gene products on cell surfaces is essential for recognition of foreign antigen by CTLs. If MHC antigens have an altered expression, such cells will successfully evade the cytotoxic immune response by CTLs.

Immunodiagnosis in Cancer

TAAs can be useful tumour markers in the diagnosis and management of various tumours. An ideal tumour marker is released only from tumour tissue, is specific for a given tumour type (to direct diagnostic assessment), is detectable at low levels of tumour cell burden, has a direct relationship to the tumour cell burden and the marker concentration in blood or other body fluid, and is present in all patients with the tumour. Most tumours release antigenic macromolecules into the circulation that can be detected by immunoassay. Although useful in monitoring patients for tumour recurrence after therapy, no tumour marker has undisputed specificity or sensitivity for application in early diagnosis or mass cancer screening programs.

Carcinoembryonic antigen (CEA) is a protein-polysaccharide complex found in colon carcinomas and in normal foetal intestine, pancreas, and liver. A sensitive immunoassay can

detect increased levels in the blood of patients with colon carcinoma, but the specificity is relatively low because positive tests also occur in heavy cigarette smokers and in patients with cirrhosis, ulcerative colitis, and other cancers (e.g., breast, pancreas, bladder, ovary, cervix). Monitoring CEA levels may be useful for detecting cancer recurrences after excision of a tumour that had been associated with elevated CEA.

α-Fetoprotein, a normal product of foetal liver cells, is also found in the sera of patients with primary hepatoma, yolk sac neoplasms, and frequently, ovarian or testicular embryonal carcinoma.

β-Subunit of human chorionic gonadotropin (β-HCG), measured by immunoassay, is the major clinical marker in women with gestational trophoblastic neoplasia (GTN)-a disease spectrum that includes hydatidiform mole, nonmetastatic GTN, and metastatic GTN and in about 2/3 of men with testicular embryonal or choriocarcinoma. The βsubunit is measured because it is specific for HCG.

Prostate-specific antigen (PSA), a glycoprotein located in ductal epithelial cells of the prostate gland, can be detected in low concentrations in the sera of healthy men. Using an appropriate upper limit of normal, assays with monoclonal antibodies detect elevated serum levels of PSA in about 90% of patients with advanced prostate cancer, even in the absence of defined metastatic disease. It is more sensitive than prostatic acid phosphatase. However, because PSA is elevated in benign prostatic hypertrophy, it is less specific. PSA can be used to monitor recurrence after prostatic carcinoma has been diagnosed and treated.

CA 125 is clinically useful for diagnosing and monitoring therapy for ovarian cancer, although any peritoneal inflammatory process can cause increased circulating levels.

Radiolabeled monoclonal antibody B72.3, which recognizes a pancarcinoma antigen (one that recognizes carcinomas from all tissues) termed TAG-72, is being used in tumour localization studies to find occult tumour deposits. The clinical benefit of finding such occult tumours is under study.

The Prospects for Immunotherapy in Cancer

The use of **vaccines** especially for virally induced cancers has become a reality as in Marek's disease of chicks. The hepatitis B vaccine, already widely in use, will be able to eliminate hepatomas. Vaccines for the Epstein - Barr virus and human T cell leukaemia virus are not, so far available, but the outlook seems promising.

Passive cellular immunotherapy

Infusions of IL-2 may prove a useful adjunct to cellular immunotherapy as they enhance T cell and NK cell cytotoxicity. Administration of lymphokine - activated killer (LAK) cells in association with IL-2 have been extensively studied in vivo and in vitro. Sometimes the cells are first exposed to phytohemagglutinin, a lymphocyte mitogen, to expand a broad variety of peripheral lymphoid cells. The availability of purified recombinant IL-2 in large quantities has made the LAK cell plus IL-2 technique feasible, and some melanoma and renal carcinoma patients have shown objective responses.

Passive humoral immunotherapy

It may be possible to use passive immunotherapy via **monoclonal antibodies**, especially against tumours showing unique tumour specific antigens. Conjugation of cytotoxic drugs,

toxins or radio isotopes to monoclonal antibodies was thought to be the exciting new way of "homing in" onto tumour cells, leaving normal cells untouched. Antilymphocyte serum has been used in chronic lymphocytic leukemia and in T-cell and B-cell lymphomas, resulting in temporary decreases in lymphocyte counts or lymph node size. Some studies of murine monoclonal antibodies against various antigens associated with malignant melanoma and lymphomas have shown significant responses; now "humanized antibodies" are used to avoid an immune reaction against mouse immunoglobulin. However, several tumour associated antigens are also displayed on normal cells and the many agents used as conjugates are too toxic for human use. The new "magic bullet" therefore, needs more research before it becomes a reality for the treatment of human cancers.

Active specific immunotherapy

Approaches designed to induce therapeutic cellular immunity in the tumour-bearing host are more promising than passive immunotherapy techniques. Inducing immunity in a host that failed to develop an effective response in the first place requires special procedures to present the tumour antigens to the host effectors. Intact tumour cells, defined tumour antigens, or general immunostimulants are used.

Autochthonous tumor cells (cells taken from the host) have been used—after irradiation, neuraminidase treatment, hapten conjugation, or hybridization with long-term cell lines in vitro—in kidney carcinoma and malignant melanoma patients, among others.

Allogeneic tumour cells (cells taken from other patients) have been used in patients with acute lymphoblastic leukemia and acute myeloblastic leukemia.

Defined tumour antigen-based vaccines are among the most promising approaches in cancer immunotherapy. An increasing number of tumour antigens have been unequivocally identified as the target of specific T cells grown from cancer patients.

Cellular immunity (involving cytotoxic T cells) to specific, very well defined antigens can be induced using short synthetic peptides in adjuvant or bound to autologous antigen-presenting cells in vitro (antigen pulsing). These antigen-pulsed, antigen-presenting cells are reintroduced intravenously and stimulate the patient's T cells to respond to the pulsed peptide antigen. Early results in clinical trials have shown significant responses.

Antigen-specific immunity can also be induced with recombinant viruses (eg, adenovirus, vaccinia virus) expressing such TAAs as CEA. These antigen-delivery viruses are being tested for anti tumour effectiveness.

Nonspecific immunotherapy

Recombinant Interferons such as (IFN-α, IFN-γ and IFN-β) have anti-tumour and antiviral activity. Depending on the dosage, IFNs may either enhance or decrease cellular and humoral immune functions and may affect macrophage and NK cell activity. Human clinical trials have indicated that IFNs have antitumour activity in hairy cell leukemia, chronic myelocytic leukemia, and AIDS-associated Kaposi's sarcoma. However, IFNs are quite toxic; patients may develop fever, malaise, leukopenia, alopecia, and myalgia.

Bacterial adjuvants (e.g, attenuated tubercle bacilli -BCG), or killed suspensions of *Corynebacterium parvum* have been used in randomized trials. They have been used with or

without added tumour antigen to treat a broad variety of cancers, usually along with intensive chemotherapy or radiotherapy. Direct injection of BCG into melanoma nodules almost always leads to regression of the injected nodules and, occasionally, of distant, noninjected nodules. Intravesicular instillation of BCG in patients with superficial bladder carcinoma has prolonged disease-free intervals, possibly as a result of immunologic mechanisms.

Other **miscellaneous factors** such as tumour necrosis factor α (TNF α) and lymphotoxin have been shown to kill tumour cells in vitro and in vivo. They also stimulate the functional activity of many types of immune cells such as CTLs, NK cells and macrophages, thereby augmenting the immune resistance to tumours. Colony stimulating factors regulate proliferation of granulocytes and macrophages, they also enhance tumoricidal activity in macrophages and induce TNF production by monocytes. These factors can also be used to treat pancytopenia, a major complication of cancer chemotherapy, since they stimulate normal haematopoietic progenitor cells in vivo.

CHAPTER – 20

IMMUNITY AGAINST INFECTIOUS DISEASES

The development of active immunity has its origins in the long and legendary fight against microbial infections and toxins. The human body lives in constant contact with bacteria, viruses, parasites and fungi. Some of these associations are beneficial, others turn sour. With the understanding of the many varied host defences against infection, was born the concept of active protection against infectious disease. This chapter deals with the mechanisms of host defence in the face of constant challenges by a shrewdly manipulative sea of micro organisms.

Immune Responses to Bacterial Infections

The cynical among us may suggest that bacteria have been, and will always remain, one jump ahead of man's endeavour to conquer infectious disease. The varied and ingenious strategies adopted by bacteria ensure their continued survival.

(a) Attachment and colonization

The mucous membranes are constantly exposed to micro organisms from the environment. To colonize the mucous membranes bacteria must have the ability to adhere closely to membrane surfaces and multiply there. It is now believed that *Streptococcus mutans* causes dental caries. The organism has a constitutive enzyme, glucosyl transferase, which is able to convert sucrose to dextran which is utilised by the organism for adhesion to the tooth surface. Attachment is most often mediated by surface receptors on both host and bacterial cells. Group A β haemolytic *Streptococci* adhere by the M antigen, the protein component of surface fimbriae. In *Escherichia coli*, the adhesive factor is a protein filament on the bacterial surface, designated the K88 antigen. Gonococci adhere to epithelial cell surfaces by pili. These are just a few examples of novel methods of attachment, some to specific receptors on human cells. Attachment ensures that the organism is not washed off by body secretions such as saliva, mucous or urine and the organism need no longer compete with normal flora for a place to pitch its tent.

Since adherence to epithelial cells of the mucous membranes is so vital to establish infection, the host has devised several mechanisms to overcome this ploy. The **secretory antibody**, IgA, affords protection in secreted body fluids such as tears, saliva, nasal and intestinal secretions. IgA prevents bacterial adherence to mucosal surfaces. If an infectious agent succeeds in dodging the IgA barrier, it is confronted with the immunoglobulin IgE, which is present in mucosal secretions. IgE mediated release of mast cell contents helps enhance the inflammatory response.

(b) Resistance to phagocytosis

The principal host defence mechanism is ingestion and destruction by phagocytosis. This is effectively overcome by the capsular material that some bacteria produce. Capsulated strains

of the pneumococcus and *Bacillus anthracis* are resistant to phagocytosis. The principal factor responsible for this resistance is the polysaccharide of the capsule of pneumococcus and the polypeptide in the capsule of *B. anthracis*.

The host defence mechanism operates to circumvent this problem by ensuring that **opsonization** by antibody or complement takes place. Opsonization greatly facilitates phagocytosis and is the basis of immunity to such infections. Opsonization with C3b attracts cells with the CR I receptor, such as primate red cells. Complexes with aggregates of red cells are then transported to the liver for phagocytosis. The lipopolysaccharide of gram negative organisms activates complement via the alternative pathway leading to cell lysis. Biologically active substances such as C3a and C5a aid chemotaxis and enhance the inflammatory response.

(c) Intracellular growth by bacteria

Some bacteria are resistant to intracellular killing. Tubercle and leprosy bacilli and the *Brucella* species are able to survive and multiply within phagocytes and are thereby easily disseminated within body tissues. These organisms defy the killing mechanism within phagocytes in a variety of ways. *Mycobacterium tuberculosis* inhibits fusion of lysosome with phagosome, *Mycobacterium leprae* has a resistant outer coat, some rickettsiae slip out of the phagosome to survive undisturbed in the cytosol.

Where antibody or complement have no access to intracellular organisms, the human immune mechanism reacts using **cellular immunity**. Both cytotoxic T cells and activated macrophages play an important part in cell mediated killing of intracellular organisms. Chronic granulomatous reaction represents an attempt by the body to wall off persistent infection. The activated macrophage has an abundance of hydrolytic enzymes and densely packed macrophages are the prominent feature of chronic granulomas.

(d) Microbial toxins and enzymes

A formidable array of toxins and enzymes are produced by micro organisms to promote their survival in the human host. The production of coagulase by staphylococci is closely related to virulence. Bacteria coated with fibrin, as a result of coagulase action, resist phagocytosis. *Pseudomonas aeruginosa* releases elastases which inactivate C3a and C4a. Organisms such as gonococci and meningococci produce proteases that split IgA dimers. Several gram negative organisms produce drug resistance enzymes which act against antibiotics. The cholera toxin attaches to a specific receptor the - GMI ganglioside, on the enterocytes, before the toxin begins to take effect.

Locally synthesized IgA prevents not only bacterial colonization, but also toxin attachment to its receptor. Hence oral cholera vaccines which promote local IgA synthesis are considered far more beneficial than parenteral IgG producing cholera vaccines.

Immune Responses to Viral Infections

Viruses escape much of the antibody mediated immune response since they are obligate intracellular parasites. Viruses attach to specific receptors on the cell surface and are initiated into the host system at several portals of entry: skin and conjunctiva, directly into the blood or via the mucous membranes lining the upper respiratory, gastrointestinal and genito urinary tracts.

Viral escape mechanisms have evolved in many ingenious ways. The adeno, entero, influenza and para influenza viruses occur as many antigenically distinct serotypes. Immunity against one serotype offers no protection against a subsequent unrelated serotype. Further, with infections such as influenza and the common cold the incubation period is short and the organ of tropism is practically at the portal of entry. There is little time or exposure of the virus to initiate or interact with the immune system. **Human interferon** seems the most significant mechanism of defence against such infections.

Antigenic shift and drift is a mechanism the influenza virus has evolved to keep the defence system at bay. By this ploy the virus is capable of constantly changing the structure of its surface proteins. This occurs by **antigenic drift** which results from point mutations in the genome of the virus; or by **antigenic shift** where there are major changes in the viral genome due to recombination between human and animal viruses. Entirely new strains of viruses thus evolve; an immune reaction against the original strain is rendered ineffective against the new strain and major epidemics have resulted.

Varicella zoster and herpes viruses remain dormant and protected in neural ganglia. Being in such privileged sites they are not exposed to serum antibody. Herpes viruses have evolved a method of spread, by cell to cell contact via intercellular bridges, hence there is no extracellular exposure.

Other viruses such as the Epstein - Barr virus have a life long association with the host, being attached to receptors on B cells.

The host ensures adequate defence in a variety of ways. The mucosa lining the tracts which communicate to the exterior is replete with lymphocytes, plasma cells and macrophages. Virus specific cell mediated immunity operates via T cells stationed for defence at mucosal sites. These T cells are also active against those viruses that spread from cell to cell and remain hidden from humoral factors. Cytotoxic T cells (CTLs) are directly cytotoxic to cells infected with viruses. Virus specific antigens of infected cells are recognized by receptors on T cells in association with MHC Class I antigens. CTL proliferation, activation and target cell cytotoxicity ensues. If cell to cell spread of virus occurs, T helper cells stimulated by antigen, secrete IFN γ, which renders contiguous cells immune to viral replication. IFN γ also enhances NK cell activity and resultant non specific killing of target cells. If viral antigen stimulates specific antibody, this antibody is used to mediate antibody dependant cellular cytotoxicity (ADCC).

Secretory IgA in mucous secretions acts locally to neutralize virus at the portal of entry, preventing attachment of virus to epithelial cells and subsequent invasion into tissues. Serum antibodies IgG, IgM and IgA neutralize viruses during their short period of viraemia, as in the case of polio and rabies virus infections. Blood borne viruses like the arboviruses, hepatitis B virus and cytomegalo virus come under antibody attack. Antibody mediated, complement induced lysis of enveloped viruses occurs. Anti neuraminidase prevents release of infectious virus and counters the spread of viral particles.

And finally the neonate is protected from lethal viral infections by immunoglobulins in breast milk and colostrum. For a comprehensive view of host defence mechanisms against viral infections see Figure 20.1.

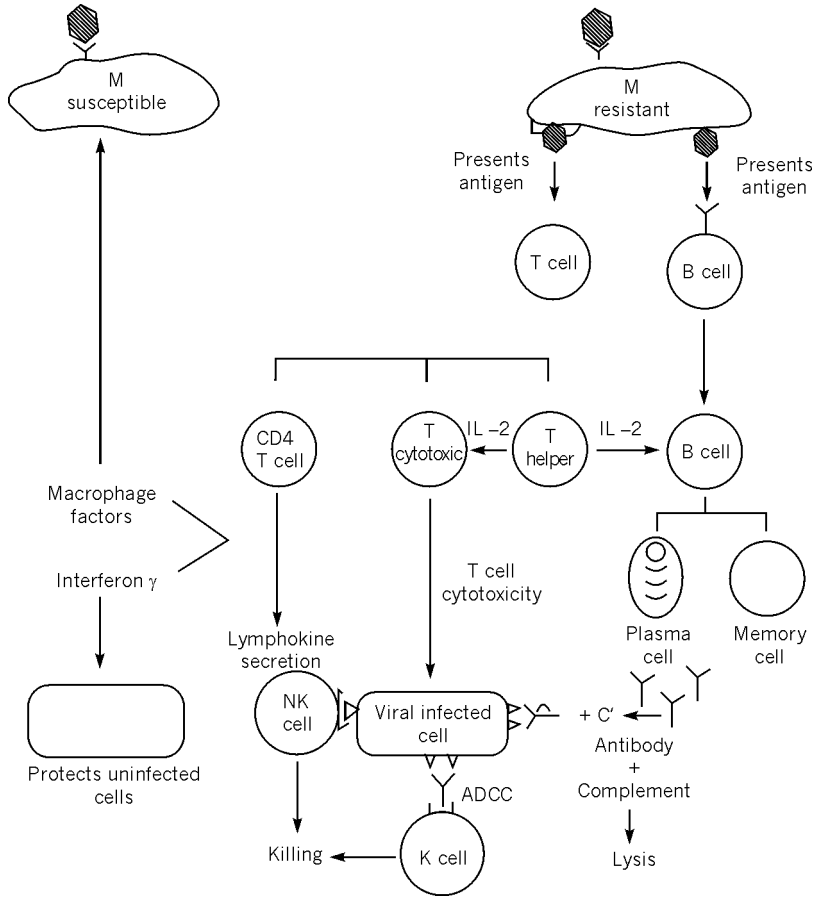

Figure 20.1. Host defence mechanisms against viral infections. Macrophages (M) may either enhance viral replication when susceptible or restrict viral growth when resistant. B cells after antigen presentation by macrophages differentiate into memory cells and plasma cells. T cells have a multifunctional role: they provide B cell help, cause cytotoxicity and secrete lymphokines.The virally infected cell is hence subjected to complement mediated lysis, T cell cytotoxicity, NK cell activity and ADCC.

Immune Responses to Parasitic Infections

Parasites are biologically complex entities, that are well adapted for survival in human hosts who are immunologically competent. Why are such hosts unable to mount an effective defence strategy against even the simplest protozoan parasites? Because there is little evidence of immunologic protection against parasitic infections, acquired specific immunity has been disputed for a long time. Micro organisms to a certain extent evade immune responses by rapid multiplication. Parasites, because of their complex life cycles require time for multiplication and therefore they have evolved various methods of evasion - so successful that parasites survive in immune hosts for years.

The many ways in which parasites block normal microbicidal mechanisms is illustrated in the following figure (Figure 20.2).

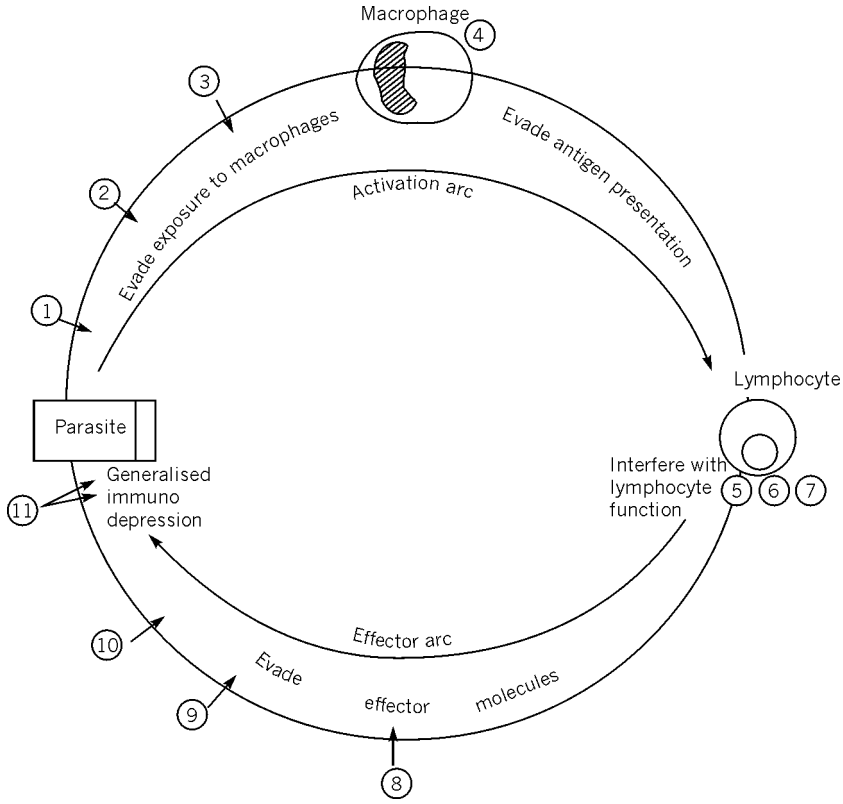

Figure 20.2. Mechanisms of immune response evasion by parasites.

1. When parasites such as the *Plasmodium* species become intrcellular and enter the liver, or when metacercariae which do not multiply in the host get embedded in the eye or the brain they escape the immune mechanism.
2. Similarly when parasites reside and thrive solely in the gut lumen there is little contact with the immune system unless tissue invasion occurs. Helminths within the gut and *Giardia lamblia* are common examples of this phenomenon.
3. Other organisms such as the Schistosomes disguise themselves with host antigens. Schistosomes exhibit glycoprotein/glycolipid antigens derived from host red blood cells as the parasites penetrate through the skin. Hence, host responses are not directed to these parasites, only to newly entered schistosomulae. This phenomenon has been termed concomitant immunity.
4. *Toxoplasma gondii* and *Trypanosoma cruzi* live within phagocytic cells. *T.gondii* inhibits phagosome-lysosome fusion. *T.cruzi* escapes from the phagosome to lie dormant in the cell cytosol.

5. Trypanosomes, leishmania and malaria parasites live within lymphocytes.
6. They inactivate host lymphocytes and cause polyclonal B cell stimulation.
7. This results in an abundance of ineffective and directionless antibodies.
8. Helminths such as the Ascaris migrate around the body stimulating various responses and then move away from an established response, escaping their consequence by entering the gut.
9. The malaria parasite exhibits stage and species specific antigens, shedding antigens at every stage. *Entamoeba histolytica* also regularly sheds its surface antigens confusing the immune system even more.
10. Antigenic variation is the best known example of the evasion tactic. *Trypanosoma brucei* is particularly successful at this gambit. These organisms display several glycoprotein surface coats each with a variable antigen type. When a response is mounted against one type, another one is manifest requiring a whole new set of antibodies.
11. Several parasites inhibit cell or antibody binding, cause depletion of antigen sensitive B cells and generalized immunodepression. *T. brucei, T. gondii,* the *Plasmodia* and *E.histolytica* are a few examples of parasites that cause generalized immunodepression.

The **host's response** to parasitic infections is not totally non existent. Innate or natural immunity plays an enormous role as evidenced by the fact that of all the protozoa (animal and human) that man comes into contact with, only few are pathogenic to humans. **Genetic factors** also play a role in host susceptibility to parasitic disease.

Antibody mediated immunity is only partially effective and only in some cases. **Premunition** is a term used to describe a controlled level of parasitemia, as in malaria, which results from antibody action. The sporozoite and merozoite stages of plasmodium evoke antibody responses that mediate premunition. The immunoglobulin IgE, represents an important line of defence. A series of IgE molecules have been found to coat worms and lead to eosinophil degranulation. The major basic protein (MBP) released, produces worm damage and other vasoactive amines enhance a local inflammatory response.

Cellular immunity via T cytotoxic cells does not appear to play a predominant role. However,T cell related lymphokines help activate the formidable macrophage, which is important in the intracellular killing of parasites such as *T.gondii, Leishmania* sp. and *T.cruzi*. Parasites have held sway over the human host, in addition, by producing damaging immunopathologic reactions such as liver granulomata and autoimmune cardiac disease.

The overall impact of host-parasite interaction seems thus to swing in favour of the parasite. This is evidenced by the finding that all attempts at successful vaccination against parasites have so far failed. It remains to be seen if man's ingenuity when pitched against the parasites' impressive array of weapons comes up the victor.

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CHAPTER – 21

IMMUNIZATION

The greatest triumph of immunology has been the successful use of immunization procedures in the control of potentially fatal infectious diseases. The concept of immunization rose from the observation that individuals who recover from certain diseases are protected for life from recurrences. The introduction of small quantities of fluid from active small pox pustules into uninfected persons (variolation) was an effort at mimicking natural infection. It is fortuitous that these experiments were done in 1721 in an age when ethical clearance was not required! Jenner introduced vaccination in 1796 using cow pox to protect against small pox. This was the first documented use of live attenuated vaccination and the beginning of modern immunization. Historical milestones in immunization are listed in Table 21.1.

Table 21.1: Milestones in Immunization

Variolation	1721
Vaccination	1796
Rabies vaccine	1885
Diphtheria toxoid	1925
Tetanus toxoid	1925
Pertussis vaccine	1925
Viral culture in chick embryo	1931
Yellow fever vaccine	1937
Influenza vaccine	1943
Viral tissue culture	1949
Polio vaccine (Salk)	1954
Polio vaccine (Sabin)	1956
Measles vaccine	1960
Tetanus immune globulin (human)	1962
Rubella vaccine	1966
Mumps vaccine	1967
Hepatitis B vaccine	1975
Licensure of first recombinant vaccine (Hepatitis B)	1986
Meningitis C vaccine	1999

Primary and Secondary Immune Responses

The first exposure to an antigen evokes a **primary response**. Immediately after introduction of immunogen little or no antibody is detected. This is called the inductive or latent period (Figure 21.1). During this period, the immunogen is recognised as foreign and processed. The duration of this period is variable and depends on the type of antigen used, species of animal and route of immunization.

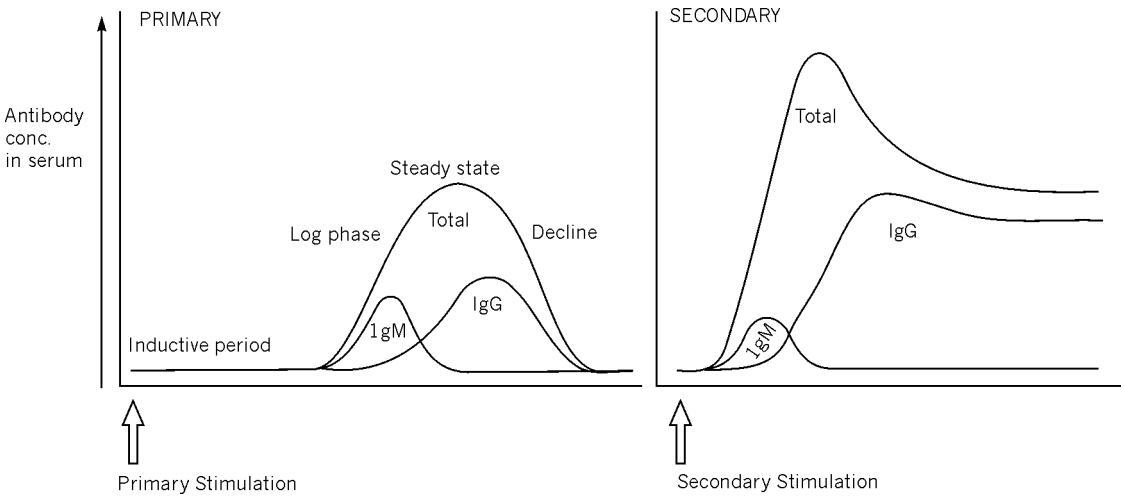

Figure 21.1. Immune responses to primary and secondary stimulation.

During the logarithmic phase, antibody concentration increases logarithmically for 4-10 days, until it reaches a peak. This peak antibody level usually takes 4 to 5 days for erythrocytes, 8-12 days for soluble proteins and 2 to 3 months for the toxoid of *Corynebacterium diphtheriae*.

The log phase is followed by a steady phase, where rates of antibody synthesis equal rates of antibody catabolism. The decline phase follows where antibody synthesis steadily falls, finally reaching pre immunization levels. The early primary response is characterized by a predominance of IgM over IgG, IgM production is transient and within 2 weeks of initiation of the response, IgG predominates.

The **secondary immune response** occurs upon second exposure to the same immunogen, weeks, months or even years later. The secondary immune response is accompanied by an accelerated response from already committed B lymphocytes in the memory pool. Rapid proliferation and differentiation into plasma cells yields a higher antibody output. The secondary response is characterized by an initial negative phase, which is due to the immediate reaction of pre existing antibody with new immunogen. An enhanced response follows due to anamnestic recall of pre committed memory cells. This enhanced response underlies the principle of administering booster doses after specific time intervals during immunization procedures. Immunologic memory can last for years, providing long lasting immunity to certain bacterial and viral infections. The evolution of immunologic memory is a function of T helper cells. T independent antigens therefore cannot elicit memory or for that matter a secondary IgG response.

The Classification of Immunity

Immunity can be classified as:

- Natural/Innate (Discussed in Chapter 2)
- Acquired immunity can be:
  - Passively acquired immunity
  - Actively acquired immunity

Passively acquired immunity

Passive immunity is acquired (*i*) by the newborn from the mother; (*ii*) by administering preformed immunoglobulins to an individual.

Maternal transfer of antibodies

The neonate is endowed with a relatively immature lymphoid system; the premature baby with a grossly ineffective immune mechanism. In early life, the newborn is thus protected by maternally derived antibodies (IgG) transferred passively via the placenta. Colostrum and breast milk also afford significant protection to the neonate. The major immunoglobulin in milk is the secretory IgA, which remains in the gut of the newborn, protecting the intestinal mucosal surfaces from enteric pathogens. Interestingly, it has been found that the secretory IgA in the breast milk is specific for bacterial and viral antigens found in the mother's gut. It is presumed that IgA producing cells responding to gut antigens migrate from the gut mucosa to colonize breast tissue, now considered to be a part of the **mucosal associated lymphoid tissue (MALT)**. Here the IgA producing cells secrete specific antibodies which appear in milk. This circuit has also been termed the **entero-mammary axis**.

Gamma globulins

Antibody, either as whole serum or concentrated gamma globulins (immune globulin), is obtained from human volunteers who have recovered from a specific infectious disease or have received immunization. The globulin consists predominantly of IgG. Administration of such human immune globulin (HIG) offers immediate protection to individuals who are at risk, particularly where active immunization may take 7-10 days for effective antibody production. Passive immunization is also useful to those individuals who are unable to produce antibody for themselves. Hazards associated with administering human immune globulin (as pooled serum) include transmission of blood borne viruses - hepatitis B or C viruses and the human immuno deficiency virus (HIV). Purified IgG preparations are, however, free of these virus particles.

Antibodies may also be available from animal sera. However, such immunoglobulins are less desirable, as non human proteins are cleared away by the host immune response against them. In addition, hazardous immune reactions against animal proteins may lead to the development of anaphylaxis or serum sickness. Neither human nor animal immune globulin should be administered intravenously for fear of anaphylactic reactions.

Human immune globulin against varicella, the varicella zoster immune globulin (VZIG) is indicated in individuals with defective immune systems such as premature infants, children with immunodeficiency diseases and patients on steroid treatment. The vaccinia immune globulin is given to patients with disseminated vaccinia or other complications of small pox vaccination. Rabies and hepatitis B immune globulin is administered to individuals who have been exposed and are at risk. Diphtheria and botulism antitoxin is used as therapy in patients who have contracted the disease. The equine source of these latter two antibodies is still widely used.

Tetanus antitoxin, now a human immune globulin, is given both prophylactically and therapeutically in appropriate cases.

Antibodies (equine) for non infectious conditions is also available: antivenin against black widow spider and snake venoms. Human immune globulin to the Rh blood group is widely used to prevent haemolytic disease of the newborn.

Active Immunity

Besides suffering the disease (and surviving it!), vaccination is the only other way of acquiring active immunity against an infectious agent. The advantages of active over passive immunization are due to the fact that the individual's immune system is stimulated to produce an immune response against a given antigen. Host participation ensures that both the humoral and cellular components of the immune system are activated. Consequently, T cell help is recruited and immunologic memory is available to boost the response after subsequent exposure to the antigen. Antibodies so formed are longer lasting as compared to passively acquired immunoglobulin.

Active immunization may be performed with either **killed** or **live attenuated vaccines** and **toxoids**.

The commonly used killed vaccines are:

- bacterial vaccines for
  - typhoid
  - cholera
  - pertussis
  - plague
- viral vaccines for
  - rabies
  - poliomyelitis (the Salk vaccine)
  - hepatitis B
  - influenza

Live Attenuated Vaccines Include

- bacterial
  - BCG – a live attenuated *Mycobacterium bovis* for tuberculosis.
  - Ty21a – live oral attenuated mutant typhoid bacillus
- viral:
  - live vaccinia virus for small pox
  - rubella
  - measles
  - mumps
  - polio (the Sabin vaccine)
  - yellow fever virus

Besides these, there are **toxoid vaccines** for:

- diphtheria
- tetanus.

Polysaccharide vaccines for

- *Haemophilus influenzae* type B (Polyribosyl-ribitol-phosphate, conjugated to tetanus protein)
- *Neisseria meningitidis* (a combination vaccine against groups A,C,Y,W135 and the new Meningitis C vaccine)
- *Streptococcus pneumoniae* (a polyvalent 23 valent polysaccharide vaccine and the new 7-valent vaccine)

Live attenuated vaccines have many **advantages**. Attenuation mimics the natural behaviour of the organism without causing disease. The immunity conferred with live attenuated vaccines is superior because actively multiplying organisms provide a sustained antigen supply. The immune response takes place largely at the site of natural infection as in the case of the live polio vaccine and the oral typhoid vaccine producing an obviously advantageous local secretory IgA response.

The **hazards of using attenuated vaccines**, though uncommon, must be documented as the risk of developing complications is a very real one. A very small number of individuals develop encephalitis following measles vaccine, however, the danger of developing encephalitis from natural infection is far greater. There is the possibility of the attenuated virus reverting to its virulent form; chances of this decrease if the attenuation incorporates several gene mutations instead of just one. Preserving adequate cold storage facilities and maintaining the cold chain from the laboratory into the field, is a continuing problem especially in the tropics. Live attenuated vaccines are not advised in patients with an immunodeficiency disease, in patients on steroid and other immunosuppressive treatment and for those undergoing radiotherapy. Malignancies such as lymphomas and leukaemias and pregnancy are all contraindications to the administration of live attenuated vaccines. The oral polio vaccine is contraindicated for any member of a household where there is a patient with a lymphoma or leukaemia, as the live virus is shed in the stool of a vaccinated individual and poses a transmission risk. Table 21.2 lists the current experimental and restricted use vaccines.

Table 21.2: Experimental and restricted use vaccines

Vaccine	*Status*
Adeno virus	Live attenuated, for military recruits
Anthrax	For those with occupational exposure; military
Arboviruses: Kyasanur Forest Disease Japanese encephalitis	Experimental only 50-60% protection Killed vaccines
AIDS	Experimental
Cholera	New oral vaccine restricted for travellers
Cytomegalovirus	Experimental
Malaria	Experimental *Plasmodium falciparum* malaria vaccine based on the circumsporozoite protein
Gram negative bacteria	Experimental and restricted
Leprosy	Heat killed *M. leprae* + BCG. Clinical trials are on
Rota virus	Live oral tetravalent vaccine, withdrawn in 1998 due to rare complication with intussusception; new vaccine trials now on.

Newer approaches to vaccine preparation

Since the process of attenuation is cumbersome and time consuming, several newer approaches to vaccine development are being researched.

(*a*) *Sub unit vaccines*

Sub unit vaccines are being designed, which use only the relevant immunogenic portions of the organism. This has been possible using monoclonal antibodies and radio labelling techniques. Surface projections of the influenza virus, the measles virus and the rabies virus elicit neutralizing antibody and can be exploited for this purpose. If the low immunogenicity of these sub units can be overcome, such vaccines are stable, free from extraneous proteins and nucleic acids and precise in their composition.

(*b*) *Biosynthesis of immunogenic proteins*

Specific immunogenic surface proteins need to be available in large quantities for vaccine preparation. The problem of isolating and characterizing antigenic protein moieties has been overcome by cloning the genes that code for these proteins in bacterial or eukaryotic (yeast) cells or in the vaccinia virus. In 1986, the first recombinant (cloned) viral vaccine was licensed for use. The Hepatitis B surface antigen (HBsAg) is the immunogen that stimulates protective immunity. The gene that codes for HbsAg has been cloned most successfully in the yeast cell. The antigen prepared by growing the yeast cells containing the recombinant gene in mass culture is widely used as a safe and effective vaccine.

(*c*) *Synthetic peptide vaccines*

A limited number of sites on an organism are involved in evoking an immune response. If these sites, consisting mainly of peptide fragments, can be synthesized they provide a possible means of obtaining chemical polypeptides as vaccines. Unfortunately, this task has been made harder by the finding that immunogenic peptides are not simple, linear sequences of amino acids and the final configuration of the protein cannot always be synthesized in the form that B cells recognize. Besides, amino acid sequences of these peptides are discontinuous and are brought together by folding of the molecule.

These and other strategies such as using anti idiotypes as vaccines are still very experimental, though animal studies have been encouraging.

CHAPTER – 22

IMMUNODEFICIENCY DISEASES

The two major arms of the immune system-**antibody (B-cell)** mediated immunity and **cellular (T-cell)** immunity, help defend the host against bacterial, viral, fungal and protozoal infections. They also perform the important functions of immune surveillance for malignant cells. In these, the many facets of defence and surveillance, they are ably aided by two other **ancillary systems: complement** and **phagocytosis**. Immunodeficiency disorders are consequently discussed under four main headings:

- Immunoglobulin (B-cell) immunodeficiency disorders
- Cellular (T-cell) deficiency diseases
- Phagocytic dysfunction
- Complement deficiency states

Immunoglobulin (B-cell) Immunodeficiency Disorders are summarised in Table 22.1

(a) X-linked infantile hypogammaglobulinaemia

X linked hypo or agammaglobulinaemia was the first immunodeficiency disorder to be described clinically. The condition is X linked and the gene governing the disorder has been localized to the long arm of the X chromosome. Because infants are born with IgG transferred from their mothers, the disease does not manifest until late in the first year of life. The effects of this condition usually appear in male infants between 9 months and 2 years of age.

The clinical course is marked by unusual susceptibility to pyogenic organisms namely, *Haemophilus influenzae*, pneumococci, streptococci, staphylococci and meningococci. The infections are more frequent and severe than those of normal children and recurrences are common. The infection is slow to respond to antibiotics and bronchiectasis and pulmonary insufficiency are common sequelae. These children, however, have normal resistance to common viral infections, fungi, and most gram negative organisms; but they are susceptible to poliomyelitis. Some children will manifest with symptoms of rheumatoid arthritis. Diarrhoea and malabsorption syndrome are common, almost always caused by *Giardia lamblia*. Death is due to a fatal syndrome, similar to dermatomyositis with neurologic involvement. In several patients with this syndrome, echoviruses have been cultured from blood, stool and cerebrospinal fluid.

Diagnosis is made by measurement of the serum level of each class of immunoglobulin. There is usually less than 100 mg/dl of IgG and levels of IgA, IgM, IgD and IgE are extremely low or undetectable. Examination of white blood cells shows a total deficiency of B cells. Tests for cell mediated immune function are normal. The lymphoid organs are characterized by a total lack of germinal follicles, B cells and plasma cells.

Treatment consists of intramuscular or intravenous administration of gamma globulin for life. Once the diagnosis is made, all subsequent male offspring of the mother or maternal aunts should be screened by immunoelectrophoresis every two months during the first year of life for their serum immunoglobulin profile. To test for female carriers of the gene, chromosomal analysis is done. The prognosis is good for patients whose condition is diagnosed and treated early.

(b) Common variable immunodeficiency

Common variable immunodeficiency (CVID) produces hypogammaglobulinaemia that does not appear to be genetically transmitted. It affects males and females equally. The condition occurs at any age, usually after puberty and is characterized by depressed levels of IgG. IgG levels are less than 200 mg/dl and other immunoglobulins are also markedly decreased. B cells are usually present but they do not function normally. Defects in cell mediated immunity are also observed.

There appear to be multiple pathogenetic causes of combined variable immunodeficiency. Defects include:

1. B cells do not respond to T cell help.
2. B cells synthesize but cannot secrete antibodies.
3. Helper T cells are absent.
4. Auto antibodies to B cells may be present.

Patients with CVID are subject to the same infections as those who have X linked hypogammaglobulinaemia; there is chronic involvement of sinuses and respiratory tract. CVID is also associated with several autoimmune-like diseases, resembling rheumatoid arthritis, idiopathic thrombocytopenia, haemolytic anaemia and neutropenia. CVID is often associated with severe malabsorption syndrome which can be caused by *G. lamblia* infection or gluten sensitive enteropathy. Chronic lung disease is a common feature. These patients cannot be treated with steroids for their autoimmune-like disease due to increased susceptibility to infection. There is generalized lymphoid hyperplasia.

Patients with CVID can have a normal life span. Women with the disease can have normal pregnancy and normal babies who will lack maternal IgG.

Treatment consists of gamma globulin administration for life and vigorous use of antibiotics during infection.

(c) Selective IgA deficiency

Selective IgA deficiency, one of the most common immunodeficiencies occurs in one of every 600 to 800 Caucasian persons. In this condition, IgA in the serum is less than 5 mg/dl, the levels of other immunoglobulins are normal. B cells bearing surface IgA are present, indicating that the problem is probably in the secretion of the IgA.

IgA deficiency is associated with many different clinical syndromes. The most frequent are those related to sinus and pulmonary infections due to bacteria and viruses. There appears to be an increase in autoimmune, gastrointestinal, allergic, connective tissue and malignant diseases.

Most patients with IgA deficiency have normal cellular immunity. Despite the fact that IgA deficiency predisposes to a variety of diseases, most patients are surprisingly healthy.

Patients with selective IgA deficiency should not be given γ globulin since they may recognize injected IgA as foreign. The classes of immunoglobulins present, will react against the injected IgA, leading to anaphylactoid reactions during subsequent injections. There is no specific replacement therapy for selective IgA deficiency. Vigorous antibiotic therapy is advocated for infections.

(d) Immunoglobulin deficiency with elevated IgM

Immunoglobulin deficiency with an elevated IgM (150 to 1000mg/dl) is characterized by low levels of IgG and IgA; IgD may also be elevated. In some cases the disease is X-linked, in others it appears as an acquired disorder, affecting both men and women. The clinical findings are similar to those seen in X-linked hypogammaglobulinaemia. In addition, there is a high frequency of haemolytic anaemia, neutropenia and thrombocytopenia. The IgG and IgA deficiency is thought to result from the lack of T cells which control IgM to IgG or IgA switching.

Treatment includes antibiotic therapy for infections and globulin administration for specific antigens.

(e) Selective deficiencies of IgM or the subclasses of IgG

Selective IgM deficiency rarely occurs in persons with normal IgG or IgA levels. This deficiency may precede the onset of CVID. Patients with selective deficiencies of the IgG sub classes have a decrease in total IgG, the degree of which depends on the sub class involved. The decrease is most profound in the case of IgG1 because 75% of all IgG is of this subclass. Patients with IgG deficiency are especially prone to bacterial infection with capsulated strains of *H.influenzae* and the pneumococcus. The diagnosis is made by the abnormal serum electrophoretic pattern and confirmed by quantitating the IgG sub classes. Such patients respond well to γ globulins.

Table 22.1 Immunoglobulin (B-cell) immunodeficiency disorders

Designation	Usual Phenotypic Expression		Presumed Level of Basic Cellular Defect	Known or Presumed Pathogenetic Mechanism	Inheritance
	Functional deficiencies	Cellular Abnormalities			
X-linked agamma-globulinaemia	Antibody	↓ B cells	Pre-B cells	Unknown. Intrinsic B-cell defect; underproduction of B cells	X-linked
Common variable immuno-deficiency (CVID)	Antibody	±↓B cells	B cells	↓T helper cells; auto-antibodies to B cells	Unknown
Selective IgA deficiency	IgA antibody	↓IgA plasma cells ±↓IgA B cells	Terminal differentiation of IgA cells impaired	Unknown	Usually unknown, frequent in families of patients with CVID

IgG deficiencies with increased IgM	Antibody	↓IgG and IgA plasma cells ↓IgM plasma cells ±↓IgM and IgG B cells	Failure of immuno-globulin class switching	Unknown	X linked, autosomal recessive or unknown
Selective deficiency of IgG sub classes	One or more IgG subtypes	↓Plasma cells ±↓Tcells	Unknown	Unknown	Unknown
κ chain deficiency	IgG (κ)	↓ κ+ Bcells	Unknown	Point mutation	Autosomal recessive
Transient hypogamma-globulinaemia	Antibody	↓Plasma cells B cells normal	Impaired terminal differentiation of B cells	↓T helper cells	Frequent in heterozygous individuals in families with various severe combined immune deficiencies
IgG heavy chain deficiency	IgG1, IgG2, IgG4, and in some cases IgE and IgA2	None	Chromosome deletion	Unknown	Autosomal recessive

Deficiencies of cell mediated (T cell) immunity are described in Table 22.2

Patients with T cell immunodeficiencies are extremely susceptible to opportunistic infections. They manifest with impaired delayed hypersensitivity responses and may be inherited or secondary to another disorder. Infections are much more likely in patients with pure T cell deficiencies than in those with pure B cell deficiencies. Innocuous organisms such as *Candida albicans* and *Pneumocystis* jirovecii cause serious disease and such patients are especially susceptible to the enteric bacteria, viruses and fungi. Vaccination with cowpox or the BCG may lead to a rapidly fatal outcome.

(a) Congenital thymic hypoplasia *(Di George Syndrome)*

Congenital thymic hypoplasia results from the lack of normal development of the third and fourth branchial or pharyngeal pouches, which leads to abnormality in the great vessels, and to the absence of the thymus and the parathyroid glands. It is not genetically transmitted and results from an intrauterine accident occurring before the eighth week of pregnancy. The absence of the thymus leads to deficiency in cell-mediated immunity in affected children.

The T cell defect in patients varies from profound to mild. These children do not exhibit delayed hypersensitivity reactions. The lymph nodes lack paracortical lymphocytes. Plasma cells are present and levels of immunoglobulin are normal. However, antibody responses to antigens are not normal, since no T cell help is obtained and secondary responses are lacking. As the patient becomes older, T cell function improves and usually by five years of age, there is no abnormality in cellular immunity.

The condition is treated with thymus transplantation in those infants who experience frequent infections.

(b) Severe combined immunodeficiency

Severe combined immunodeficiency (SCID) disease is characterized by marked depletion of the cells that mediate both B cell and T cell immunity. SCID is invariably fatal if left untreated. There are at least 5 variants of SCID (Table: 22.2). SCID is transmitted either as an autosomal recessive trait or an X-linked recessive trait: (*i*) Many of the cases inherited in an autosomal recessive manner are caused by a deficiency in the enzyme adenosine deaminase (ADA). (*ii*) Other patients with an autosomal recessive form of SCID lack the enzyme purine nucleoside phosphorylase (PNP). (*iii*) Another invariant of SCID is reticular dysgenesis, which is a severe combined immunodeficiency with generalized granulocyte deficiency. Newborns with this disease lack granulocytes in the blood and bone marrow and die of infection in the first few days of life (*iv*) In rare cases the common type of SCID affects the long bones and causes short limbed dwarfism and, (*v*) A form of SCID in which immunogobulins are normal,was formerly called Nezelof's syndrome, but is now termed SCID with B cells.

Clinically, onset of infections occurs at 3 to 6 months of age: chronic pulmonary infections, diarrhoea, moniliasis and failure to thrive are the most common manifestations of SCID. No tonsils are observed on physical examination and the lymph nodes are small to absent despite chronic infections. The thymus is absent or vestigial. There is a complete absence of T cells and antibody responses are low.

The pathogenesis of the common type of SCID is not known but is thought to be due to a deficiency in the enzyme - ADA. Lymphocytes lacking ADA have excessive dATP which blocks the enzyme required for making the building blocks of DNA. SCID caused by ADA deficiency can be diagnosed prenatally by amniocentesis, because fibroblasts in the amniotic fluid also show the enzyme defect.

SCID due to ADA deficiency can be successfully treated with bone marrow transplantation. Infusions of purified adenosine deaminase have also been successful.

(c) Wiskott - Aldrich syndrome

Wiskott - Aldrich syndrome is an X-linked recessive disease affecting boys and is characterized by eczema, thrombocytopenia, increased susceptibility to infection and bloody diarrhoea. These patients display anergy to common skin tests using bacterial and fungal antigens. They lack isohaemagglutinins and cannot make antibody to polysaccharides. Antibody to protein antigens is evident. Total IgG levels are normal, IgE and IgA levels are high, IgM is low. These patients catabolize their immunoglobulins faster than normal individuals. The paracortical areas of the lymph nodes are depleted of lymphocytes.

The disease is attributed to a morphological abnormality of lymphocytes. The condition can be treated with bone marrow transplantation.

(d) Immunologic deficiency with ataxia telangiectasia

Ataxia telangiectasia is a progressive neurologic disease that begins in early childhood. It is characterized by cerebellar ataxia, followed by increasing tremor and deterioration of mental function. The disease is associated with defects in cell mediated immunity and with immunoglobulin deficiencies. It is inherited as an autosomal recessive trait.

Phagocytic Dysfunction Diseases

Primary or intrinsic phagocytic disorders are related to enzymatic deficiencies within the metabolic pathway in the phagocyte, necessary for killing bacteria. Susceptibility to infection in these disorders may range from mild to overwhelming and fatal. They are susceptible to bacterial infection and fungal infection strikes the more serious cases. They have no difficulty with viral or protozoal infections.

(a) Chronic granulomatous disease

Chronic granulomatous disease is an X-linked disorder which manifests in the first two years of life. Patients are susceptible to infections with unusual organisms, normally of low virulence, such as *Staphylococcus aureus*, *Serratia marcescens* and *Aspergillus* spp. Patients present with draining lymphadenitis, hepatosplenomegaly, pneumonia, osteomyelitis and abscesses.

Due to intracellular enzyme deficiencies in the granulocytes, metabolism is impaired resulting in decreased oxygen consumption, diminished production of hydrogen peroxide and super oxide anion. The result is that intracellular killing of bacteria and fungi is impaired. Treatment consists only of treating the various infections, white cell infusions have been attempted in some cases.

(b) Specific enzyme deficiencies

(*i*) **Glucose-6-phosphate dehydrogenase** is completely lacking in the leukocyte, and produces a disease syndrome similar to chronic granulomatous disease. The disease has a later onset, affects both males and females and haemolytic anaemia is present.

(*ii*) Deficiency of leukocyte **myeloperoxidase**, needed for normal intracellular killing leads to recurrent candidial and staphylococcal infections. The leukocyte respiratory burst and super oxide anion formation are, however, normal.

(*iii*) Modest reduction of leukocyte bactericidal activity has been associated with the deficiency of leukocyte **alkaline phosphatase**.

(c) Chediak-Higashi Syndrome

Chediak-Higashi syndrome is a multi system autosomal recessive disorder. The patient presents with recurrent bacterial infections, hepatosplenomegaly, partial albinism, central nervous system abnormalities and a high incidence of lymphoreticular cancer. The basic defect appears to be abnormal intracellular killing of organisms and large granular inclusions in white blood cells are evident. The killing defect consists of delayed killing time even though "respiratory burst" and oxygen consumption are normal. Several leukocyte enzymes and microtubule function appear to be deficient.

The prognosis is poor, most children do not survive their childhood.

(d) Lazy Leukocyte Syndrome

Patients with defective neutrophil chemotaxis in association with neutropenia have an abnormal in vivo inflammatory response. Such patients are susceptible to severe bacterial infections. The prognosis is unknown.

Table 22.2: Deficiencies of cell mediated (T cell) immunity

Designation	Usual Phenotypic Expression		Presumed Level of Basic Cellular Defect	Known or Presumed Pathogenetic Mechanism	Inheritance	Main Associated Features
	Functional deficiencies	Cellular abnormalities				
Congenital thymic hypoplasia (Di George syndrome)	CMI, impaired antibody	↓ T cells	Thymus	Embryopathy of the 3rd and 4th pharyngeal pouch areas	Usually not familial	Hypopara-thyroidism, abnormal facies, cardio-vascular abnormalities
Severe combined immuno-deficiency (SCID)	CMI, antibody	↓T cells ↓ B cells	LSC	Unknown	Autosomal recessive or X linked	
(*i*) Adenosine deaminase (ADA) deficiency	CMI, antibody	↓ T cells ±B cells	LSC or early T cells	Metabolic effects of ADA deficiency	Autosomal recessive	
(*ii*) Purine nucleoside phosphorylase (PNP) deficiency	CMI ± antibody	↓ T cells	T cells	Metabolic effects of PNP deficiency	Autosomal recessive	Hypoplastic anaemia
(*iii*) Reticular dysgnesis	CMI, antibody, phagocytes	↓ T cells ↓ B cells ↓phagocytes	HSC	Unknown	Autosomal recessive	Neutropenia
Wiskott Aldrich syndrome	Antibody to certain antigens (mainly) polysacchar-ides), CMI (progressive)	↓ T cells ↓ B cells (Progressive)	Unknown	Defect in cell membrane glycoproteins	X linked	Thrombocytopenia, eczema, lympho-reticular cancers
Immuno-deficiency with ataxia telangiectasia	CMI, antibody (partial)	↓ T cells ↓ plasma cells (mainly those cells producing IgA, IgE ±IgG)	Early T cells and defective terminal differentiation of B cells	Unknown, faulty thymic epithelium, DNA repair defect	Autosomal recessive	Cerebellar ataxia, telangiectasia, chromosomal abnormalities, raised serum alpha-feto protein levels
MHC class II	CMI ±antibody	None	T cells B cells and antigen presenting cells	Defect of promoter binding protein	Autosomal recessive	Intestinal malabsorption

CMI: Cell mediated immunity LSC: Lymphocytic stem cell
HSC : Haematopoietic stem cell NK: Natural killer

Complement Deficiency States

A variety of complement deficiencies and abnormalities of complement function have been associated with increased susceptibility to infection. Complement factors are necessary for opsonization, bacterial killing and chemotaxis. Many complement related disorders are associated with increased incidence of autoimmune disease. The complement component deficiencies and the syndromes they produce are presented in Table 22.3.

Table 22.3: Complement related abnormalities and immunodeficiency

Complement deficiency	*Clinical syndromes*
C1q	Systemic lupus erythematosus (SLE)-like syndrome. Increased susceptibility to bacterial infections
C1r and C1s	SLE like syndrome. Increased susceptibility to bacterial infections
C2	SLE like disorders, anaphylactoid purpura, dermatomyositis and increased susceptibility to bacterial infections. Chronic renal disease. Antibodies to DNA present.
C3	Increased susceptibility to pyogenic bacterial infections and nephritis
C4	SLE like syndrome. Diminished chemotactic and opsonic activity and impaired antibody responses.
C5 dysfunction and deficiency	Defective chemotaxis leading to diarrhoea, recurrent bacterial infections and failure to thrive
C6	Repeated episodes of meningococcal and gonococcal infections
C7	Susceptibility to meningococcal and gonococcal infections, autoimmune disease.
C8	Disseminated gonococcal and meningococcal infections
C9	Haemolytic complement activity is reduced. No clinical abnormality
C-1 inhibitor	Results in hereditary angioedema; recurrent attacks of non-pitting oedema of skin, gastrointestinal and respiratory tracts. Laryngeal oedema can lead to respiratory obstruction and death. Jejunal oedema produces abdominal cramps and vomiting; colonic involvement produces watery diarrhoea. Attacks may be induced by tissue trauma such as dental extraction.

From Table 22.3, it can be seen that C3 is crucial for controlling bacterial infections. C3 is required for opsonization and for the ongoing pathway to C5 which is a vital chemotactic agent. Individuals with inherited deficiencies of the classical pathway ie. C1, C4 and C2 exhibit increased incidence of auto immune disease rather than infectious disease, demonstrating clearly that

the alternative pathway is capable of taking care of host defence on its own. The classical pathway,which interacts with antibody, augments clearance of immune complexes. An impairment of this function due to complement deficiency predisposes to immune complex deposition and consequent autoimmune disorders.

Acquired and Secondary Immunodeficiency

A variety of disorders are associated with secondary immunodeficiency (Table 22.4). The prototype secondary immunodeficiency disorder is the Acquired Immunodeficiency Syndrome (AIDS), caused by the human immunodeficiency virus (HIV). This will be discussed in detail in Chapter 23.

Table 22.4: Disorders associated with secondary/acquired cellular deficiency

1. Chromosomal disorders:
   Down's syndrome, Fanconi's syndrome
2. Infective disorders: HIV, Lepromatous leprosy,
   Epstein-Barr virus, chronic mucocutaneous candidiasis,
   secondary syphilis, other viral and parasitic diseases
3. Neoplastic disorders: Thymoma, Hodgkins disease and other lymphomas, any advanced malignant disease
4. Connective tissue disorders: SLE, advanced rheumatoid arthritis
5. Physical agent induced: Burns, X-irradiation
6. Other conditions: sarcoidosis, malnutrition, aging, inflammatory bowel disease, intestinal lymphangiectasia, renal failure, intravenous drug use
7. Iatrogenic causes: Chemotherapy, radiotherapy, post-surgery.

Clinical Tests Used to Assess Immune Function

Tests for immune function are required to diagnose primary and secondary immunodeficiencies. Primary immunodeficiency states are rare and often fatal. Secondary immunodeficiency states are associated with certain disease states such as diabetes and are a complication of immunosuppressive therapy, chemotherapy for malignancy, organ transplantation and treatment for autoimmune diseases. The balance between immunosuppression and fatal opportunistic infections may be guided by such tests.

Evaluation of B-lymphocyte function

The initial screening test for B-lymphocyte function is the **measurement of serum immunoglobulines**. Neither serum protein electrophoresis, immunoelectrophoresis, nor immunofixation electrophoresis are sufficiently sensitive or quantitative to be useful. Quantitative measurements of serum IgG, IgA and IgM will identify patients with panhypogammaglobulinemia as well as patients who have a deficiency of an individual class of immunoglobulin, such as selective IgA deficiency. In a patient in whom there is a strong suspicion of a humoral immunodeficiency based on clinical grounds, the total IgG may be normal. However, quantitative measurements of individual IgG subclasses may show deficiencies.

In addition to the measurement of serum immunoglobulin concentrations, some **assessment of antibody function** is a necessary part of the evaluation of humoral immunity. Antibody titers after immunization with protein antigens (e.g., tetanus or diphtheria toxoids)

and polysaccharide (e.g., pneumococcal capsular polysaccharides) are most convenient. It should be emphasized, however, that immunization with live viral vaccines should be avoided whenever an immunodeficiency is suspected. If immunoglobulin levels and/or antibody titers are decreased, the evaluation should proceed with more advanced tests of B-lymphocyte numbers and function such as lymphocyte phenotyping using flow cytometry.

Evaluation of T-lymphocyte function

Testing for defects in T-lymphocyte function is relatively difficult because of the lack of inexpensive and reliable screening tests. **Delayed type hypersensitivity (DTH) skin tests** using a panel of ubiquitous antigens can be used as a screening test in older children and adults. The presence of a positive DTH skin test generally indicates intact T-cell function and cell mediated immunity.

Disease states that produce poorly reactive skin tests

- Immune deficiency states:
  - congenital
  - acquired as in AIDS
- Malignancy
- Liver or kidney disease
- Overwhelming infections – viral
  - bacterial, such as tuberculosis
  - fungal
- Extremes of age
- Malnutrition

However, there are some important limitations to DTH skin testing. A positive DTH skin test to some antigens does not ensure that the patient will have normal cell mediated immunity to all antigens or microorganisms. For example, patients with chronic mucocutaneous candidiasis may have a limited defect in which cell mediated immunity may be intact to a wide variety of microorganisms except to candida. Furthermore, some normal individuals may have transiently depressed DTH reactions during certain viral infections. And finally, a positive DTH skin test requires prior exposure and sensitization to the antigen. Infants and young children may not have had sufficient prior exposure to have developed positive DTH skin tests.

Thus, negative DTH skin tests may not necessarily reflect abnormal T-lymphocyte function. Indirect information about T-lymphocyte function may be obtained by **enumerating peripheral blood T-lymphocytes using monoclonal antibodies**. With the help of monoclonal antibodies to stage specific T cell antigens (total T-lymphocytes -CD2 or CD3; T-helper lymphocytes -CD4; and T-cytotoxic lymphocytes-CD8); various populations of T cells can be defined. Specific monoclonal antibodies are used to react with T cells at different stages of maturation and to identify the different fractional subsets. The test is done by immuno fluorescence or immuno peroxidase staining. Defining and counting populations with the CD4+ and CD8+ marker yields information on T helper/T cytotoxic cell ratio which is normally, T helper: (65%) and T cytotoxic: (35%). This ratio is altered in certain disease states such as AIDS.

More specialized tests of T-cell function include an assessment of **lymphocyte proliferation** in response to nonspecific mitogens (e.g. phytohemagglutinin), specific antigens (e.g., candida) and/or mononuclear cells from an unrelated, histo-incompatible individual (mixed leukocyte reaction).

It is also possible, in specialized laboratories, to measure the production of a number of different **cytokines** that are involved in T- and B-lymphocyte regulation (e.g. Interleukin 2, interferon-gamma).

Evaluation of phagocytic function

The evaluation of phagocytic cells generally entails assessment of both their number and their function. For example, disorders such as congenital agranulocytosis or cyclic neutropenia are characterized by reductions in phagocytic cell number in the peripheral blood and, therefore, can be detected by using a **white blood cell count and differential**.

Assessment of **phagocytic cell function** requires a number of different assays. In vitro assays of directed cell movement (chemotaxis), ingestion (phagocytosis), and intracellular killing (bactericidal activity) are available but usually require specialized laboratories. Importantly, there are simpler assays that indirectly assess phagocytic killing by measuring the metabolic events which accompany and/or are responsible for intracellular killing. The most common of these assesses the ability of phagocytic cells to respond with an oxidative burst by measuring the reduction of nitroblue tetrazolium (NBT test).

Evaluation of the complement system

Most of the genetically determined deficiencies of the classical activating pathway (C1, C4 and C2), of C3, and of the terminal components (C5, 6, 7,8, and 9) can be detected by using antibody sensitized sheep erythrocytes in a total haemolytic complement assay since this assay requires the functional integrity of C1 through C9. Deficiencies of alternative pathway components Factors D, H and I and properdin can be detected by a haemolytic assay that uses unsensitized rabbit erythrocytes which are potent activators of the alternative pathway. The identification of the individual component which is deficient, rests on specialized functional and immunochemical tests which are specific for each component.

CHAPTER – 23

IMMUNOLOGY OF HIV INFECTION

The HIV pandemic has emerged as the single most defining occurrence in the history of infectious diseases of the late 20th and early 21st centuries. The immunopathologic effects of HIV infection are directly related to the interaction of the virus with a receptor (CD4 surface molecule) on CD4+ T cells or T helper cells. The CD4+ molecule is expressed also on the surface of monocytes, macrophages and certain neurons and glial cells from particular areas of the brain, albeit with much less density. In addition to the CD4 receptor present in macrophages, monocytes, and T cells, macrophage tropic strains of HIV 1 also need the presence of a CCR5 receptor on the cell surface to cause infection. Another receptor, CXCR4 a chemokine receptor enhances binding and internalisation of lymphotropic HIV.

The immunologic abnormalities therefore result from interference in the normal functioning of these CD4 bearing cells. The cardinal manifestation of HIV infection is the depletion of the CD4+ T cell population. Virtually all the immunologic abnormalities in AIDS can be ascribed to defective functioning by T helper cells.

Natural Course of Infection

HIV enters the body and binds to dendritic cells which carry the virus to T helper or CD4+ T cells in lymphoid tissue establishing the infection. HIV specifically targets and binds to the CD4+ T-helper cells and macrophages. After infection, replication of the virus occurs within the T-helper cells. The cells are lysed and new viruses are released to infect more T-helper cells. During the course of the disease massive numbers of virus (>1 billion/day) are released. T- helper cells are infected, and rapidly destroyed both by virus and by cytotoxic T cells. T-helper cells are replaced with nearly a billion produced per day. Over many years (average may be 10), the T-helper cell population is depleted and the body loses its ability to mount an immune response against infections. Thus, we mount a very strong immune response against the virus for a long time, but the virus is produced at a very high rate and ultimately overcomes the ability of the immune system to respond.

The T helper functions that are ablated due to HIV infection are:

- Activation of macrophages
- induction of B cell function
- induction of cytotoxic T cell function
- induction of natural killer (NK) cell function
- secretion of interleukins and other chemokines for other lymphoid cells
- secretion of haematopoietic colony stimulating factors and
- secretion of factors that induce non lymphoid cell function.

A summary of the immunologic abnormalities in patients with AIDS is given in Table 23.1.

Table 23.1: Immunologic Abnormalities Associated with AIDS

Immunologic function	*Abnormality*
Humoral functions	• Elevated serum immunoglobulins predominantly IgG and IgA in adults,including IgM in children. • Increased spontaneous immunoglobulin secretion by individual B cells. • Decreased ability to mount a de novo antibody response to a new antigen. Elevated serum levels of immune complexes.
Cellular functions	• Lymphopenia • Selective T cell deficiency with reduction in the CD4+ T cell subset (the helper-inducer sub set). • Decreased or absent delayed cutaneous hypersensitivity reactions. • Decreased in vitro lymphocyte proliferative responses to antigens and mitogens. • Decreased T cell mediated cytotoxicity. • Decreased NK cell activity.

Understanding the CD4+/ CD8+ test results

CD4+ and CD3+ counts

The normal CD4+ or helper T cell count is somewhere between 500 and 1500 cells per cubic millimeter of blood. In the absence of anti-HIV treatment, the CD4 cell count decreases, on average, about 50 to 100 cells each year. Opportunistic infections such as *Pneumocystis jirovecii* pneumonia (PCP) can occur if the CD4 count falls below 200. A large number of other infections can occur if it drops below 50 to 100 cells. Please note that the CD3+ count represents both the CD4+ and CD8+ sub sets since both these lineages carry the CD3+ marker. The CD3+ % is the percentage of CD3+ cells within the total lymphocyte count. The CD4+/CD3+ or CD8+/ CD3+ percentages are the percentage of each subset within the pool of CD3+ T cells.

CD4+ %

In healthy adults, the number of CD4+ cells make up between 32% and 68% of the total number of lymphocytes – which would include CD4+ cells, CD8+ cells and B-cells. The CD4 percentage is sometimes a more reliable measurement than the CD4+ count because it tends to vary less between measurements. For example, one person's CD4+ count may vary between 200 and 300 over several months while their CD4+ percentage remains constant at, say, 21%. Provided that the CD4+ percentage stays at 21% or higher, the immune system still appears to be functioning properly, regardless of what the CD4+ count is. At the same time, a CD4+

percentage at or below 13% – regardless of what the actual CD4+ count is – usually means that the immune system is damaged.

CD8+ count and the CD4+/CD8+ ratio

CD8+ cells, also called cytotoxic T cells, play a major role in fighting infections such as HIV. A healthy adult usually has between 150 and 1,000 CD8+ cells per cubic millimeter of blood. Unlike CD4+ cells, people living with HIV tend to have higher-than-average CD8+ cell counts. Laboratory reports also list the T-cell (CD4+/CD8+) ratio, which is the number of CD4+ cells divided by the number of CD8+ cells. Since the CD4+ count is usually lower than normal in people living with HIV, and the CD8+ count is usually higher, the ratio is usually low. A normal ratio is usually between 0.9 and 6.0. Once anti-HIV therapy is started, an increase in the T-cell ratio (i.e., a rising CD4+ count and a falling CD8+ count) is a sign that drug treatment is working. See Figure 23.1 to see what a CD4+ test result looks like.

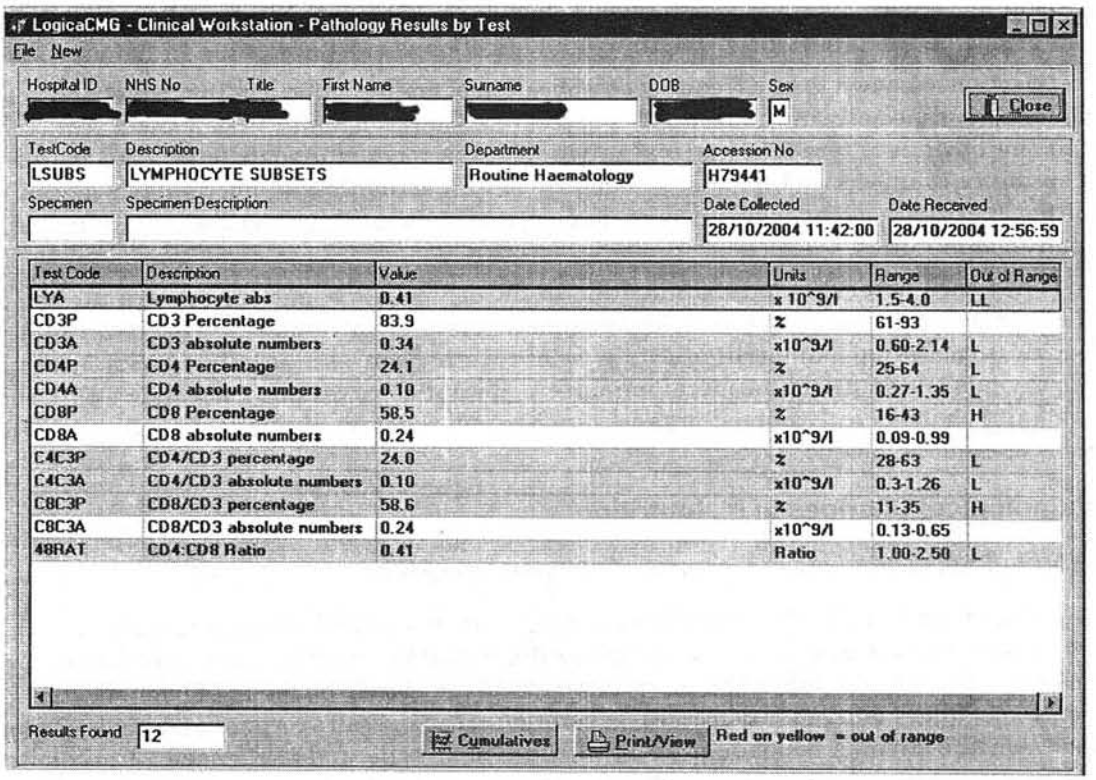

Figure 23.1 The CD4+ test result.

CHAPTER – 24

IMMUNITY AND MALNUTRITION

It is now a widely acknowledged observation that malnutrition is directly linked to immunodeficiency. The most susceptible segments of a given population are infants and the elderly where infection, nutritional deficiency and impaired immunity are frequently encountered and may be causally related. Migrant workers and their families in large metropolitan centres, living in over-crowded slums pose a major public health problem where the interplay between nutrition, immunity and infection is most evident.

Infection is known to be a frequent complication of malnutrition leading to high morbidity and mortality especially in children. This association between malnutrition and infection has led to the obvious inference that malnutrition leads to immunodeficiency and increased susceptibility to infection. Infection in itself causes an actual loss of nutrients as a result of vomiting, diarrhoea, a tendency to poor or inadequate feeding and various dietary fads prevalent in many communities.

It has been observed that malnutrition in the critical early months of intra uterine life could have far reaching effects on a child's immune system. Intra uterine growth retardation either due to maternal malnutrition or to a variety of other causes is associated with involution of the thymus and impaired neonatal immunity. Recent observations have shown that there are definite changes in lymphoid organs, number and function of lymphoid cells and in the efficacy of humoral defence factors.

Immunological Changes in Malnutrition

(a) Morphological changes in the immunocompetent organs

The thymus and other lymphoid organs react more severely to nutritional deficits than do other tissues. Protein energy malnutrition results in marked histological changes within the thymus: there is a reduction in the size and weight of the gland, depletion of lymphocytes, loss of cortico-medullary differentiation and degeneration of the Hassall's corpuscles. Similar changes are seen in the thymus dependant areas of the lymph node and the spleen. Concomitant infection such as measles produces additional lymphoid atrophy. It is possible that nutritional deprivation during intra uterine growth or in the neonate may produce irreversible morphological changes in the thymus and thymus-dependant areas of other lymphoid organs.

(b) Changes in T-lymphocyte function

Immune responses: both cellular and humoral depend heavily on efficient T cell function. Lymphoid atrophy and impaired maturation are evidenced in malnutrition. Studies have shown that 15% of children with moderate to severe protein-energy malnutrition show lymphopenia. This is said to be due to impaired differentiation in the thymus due to decreased thymic hormone activity.

Delayed cutaneous hypersensitivity reactions following challenge with common antigens are decreased in protein-energy malnutrition and improve with oral or intravenous feeding with protein and calorie rich mixtures. The mechanisms underlying this deficit are unknown. A combination of factors such as antigen recognition, processing, efficient functioning of T lymphocytes, release of lymphokines and mobilization of polymorphs and macrophages may influence the outcome of a skin test in malnourished individuals. Lymphocyte blast transformation and lymphocyte mediated cytotoxicity are both abrogated in severe malnutrition and intraepithelial T cell populations are reduced.

Changes in T cell mediated immune responses in malnutrition are rapidly corrected after nutritional supplementation, unless the nutritional insult occurs during intra uterine life or in the neonate.

(c) Changes in B lymphocyte function

The number of circulating B lymphocytes remains unchanged during malnutrition. Serum immunoglobulins are normal or modestly elevated in malnutrition and is probably a response to infection.

Antibody response to infectious agents is generally normal in protein energy malnutrition. However, if an antigen requires T cell help, and many infectious agents do, then antibody response to repeated infections is less than satisfactory; since memory is a T cell function. Impaired antibody responses improve when nutritional supplementation is adequate.

The finding that decreased secretory IgA (sIgA) response occurs over mucosal surfaces in malnutrition is of immense fundamental and applied interest. sIgA responses in nasopharyngeal and other external secretions is low and specific IgA production during vaccination with measles and polio viruses is markedly reduced. These alterations may be due to decreases in IgA bearing cells or due to a lowered turnover of the IgA secretory component from an atrophied mucosal epithelium.

(d) Altered polymorphonuclear function

Although the total number of polymorphs and leucocytes is unchanged, there are significant alterations in polymorph function during malnutrition. When nutritional deficiency is complicated by infection, chemotactic migration of neutrophils is markedly reduced; ingestion is normal but intra cellular killing of bacteria and fungi is deficient. Following phagocytosis, in the malnourished individual, the neutrophil does not exhibit the characteristic “respiratory burst” and the activity of the hexose monophosphate shunt does not rise. Similar changes are seen in iron deficiency anaemia. These abnormalities are reversed within a few weeks of nutritional supplementation.

(e) Complement function

Several studies have demonstrated consistent changes in the complement system during malnutrition. Many of the complement components are produced by the liver which is often affected as a result of protein deprivation. Undernourished children show reduced levels of C3, C1, C2 and C5 and the total haemolytic activity is reduced. There is some evidence to show that the alternative pathway may also be affected during malnutrition.

(f) Other factors

There are marked changes in several non-specific host defence factors during nutritional deprivation. Lower levels of lysozyme are found in plasma, tears, saliva and other secretions. Metaplasia of mucosal epithelia, deficient mucus trapping and cilial movement during malnutrition also influence susceptibility to infection. Interferon production is reduced during nutritional deficiency.

The interactions between nutrition, immunity and infection are important determinants of morbidity in malnourished individuals. Nutritional modulation of the immune response may influence the outcome of an infectious episode especially in the elderly, during post operative recovery, in cancer patients and other debilitating disease states and more importantly in premature and low birth weight infants. In addition, current hypothesis favours the use of tests for immunocompetence as a functional index of nutritional status. As a prognostic test for nutritional rehabilitation and disease vulnerability these assays are both reliable and sensitive.

Index