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SCHOOL OF BIO AND CHEMICAL ENGINEERNG

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – Introduction to Immunology and Immune System – SBTA1402

Introduction to Immunology and Immune System

History of Immunology

The word “immunity” (L: *immunis* - free of) was used in the context of being free of the burden of taxes or military conscription. The history of immunology is really slightly more than 100 years if you consider Louis Pasteur as the “Father of immunology” as most do. If one thinks about cellular immunology, the real history begins in the late 1950s.

From early writings, it is clear that primitive man knew about disease and its ravages . One finds in the Babylonian Epic of Gilgamesh (2000 B.C. - Mesopotamian hero) records of the presence of pestilence and disease. In other, more recent writings from old dynasties of ancient Egypt, one finds even more descriptions of disease. Further, one can even identify the disease of which they spoke. Recall that, in those days, disease and pestilence was punishment rendering as a result of bad deeds or evil thoughts. Even the old testament is filled with pestilence that God wrought upon those who crossed him. From these writings, it is equally apparent that man knew that once he had been afflicted with disease, if he survived, he was normally not able to contract it again.

The science of immunology grew from the common knowledge that those who survived many of the then common infectious diseases, rarely contracted that disease again. This was an observation that was made long before the establishment of the germ theory of disease. Louis Pasteur and Robert Koch were instrumental in defining microorganisms as the etiological agents of a large number of diseases.

In 430 B.C., Thucydides recorded that while the plaque was raging in Athens, the sick and dying would have received no attention had it not been for those individuals who had already contracted the disease and recovered and recognized their immune status. Beginning around 1000 A.D., the ancient Chinese practiced a form of immunization by inhaling dried powders derived from the crusts of smallpox lesions. Around the fifteenth century, a practice of applying powdered smallpox "crusts" and inserting them with a pin or poking device into the skin became commonplace. The process was referred to as **variolation** and became quite common in the Middle East. However, the primary intent of variolation was that of preserving the beauty of their daughters and no mention was made of saving lives.

From Turkey, the process of variolation can be traced to the inhabitants of a country called Arcassia. The people that populated this land were poor but were blessed with large number of beautiful women, which unfortunately was the chief trade and very important to the Arcassian economy. Most sales were to the Sultan of Turkey.

Eventually, the process was popularized in Great Britain, largely through the efforts of Mary Wortley Montagu. It was vehemently opposed by the Church and was highly discouraged, particularly if one were Christian. The clergy stated that it could only work of an "UnChristian" who was an infidel in the eyes of the Lord.

To complicate matters more, because there was no standardization of the inoculum engrafted, the practice occasionally resulted in death/disfigurement from smallpox. This coupled with the widespread acceptance of herbal medicine resulted in it not being widely accepted. In 1721, Mary Wortley Montagu's daughter was the first person to be engrafted in Great Britain

The first step to a safer procedure was to substitute material derived from the lesion of a cowpox (vaccinia) for the inoculation. Cowpox is a benign disease due to infection with a virus closely related to the smallpox (variola) virus.Some notable events in "early" Immunology

1774- Benjamin Jesty, a farmer who inoculated his wife with the vaccinia virus obtained from farmer Elford of Chittenhall, near Yetminster. First record of anyone using vaccinia virus to "protect" against smallpox.

In 1798, Jenner inoculated a young by named James Phipps with material obtained from a cowpox lesion. The results were conclusive but were met with great resistance by the Church. Ignorance can be hard to combat!! For this feat, Jenner received a cash prize of 30,000 pounds . The acceptance of Jenner's thesis was strengthened when 70 of the principal physicians and surgeons of London threw their weight behind him. He was elected to membership to all of the learned societies throughout Europe with the **exception** of the College of Physicians. They required that he pass an examination in Classics which Jenner refused.

To further advance the fledgling science of immunology required the development of the **Germ theory of disease.** It is here that Louis Pasteur played a pivotal role in the evolution of the science. While Pasteur's work at the Pasteur Institute in Paris was concerned with bacterial infectious disease, he was most concerned with the *prevention* of diseases that bacteria caused and how the human body was changed subsequent to infection so as to resist further insults. **Louis Pasteur**became the first experimental immunologist.

Pasteur proceeded to develop valid methods for immunization.

His first quest was with the disease chicken cholera. It was known that chicken cholera was due to infection with the "chicken cholera bacillus". Pasteur had a flask of the organism which he inadvertently left on the bench over the summer (I guess he took "summer breaks"). When Pasteur again turned his attention to the organism, he found that the culture had lost its ability to cause disease in the chicken. Briefly, this is what Pasteur did. Desiring to infect several chickens, he took his "old but viable" culture of chicken cholera bacillus and injected 8 chickens with it. Surprising, the chickens did NOT die in the usual period of time. In fact, they did not even get sick! Somewhat vexed by the loss of time, he prepared a new stock of bacteria and

re-injected the original 8 AND 10 new fresh chickens (he had to make up for lost time). After 48 hours, the 10 newly injected chickens were "sick and dying" while the original 8 were the "picture of health". Pasteur envisioned that somehow the original 8 chickens had been "changed" by the old culture and were no longer susceptible (he tried three more times to kill those stupid 8 chickens but they never got sick).

As a result of his work, Pasteur said that the virulent chicken cholera bacillus had become **attenuated** by sitting on the bench over the summer months. The similarity between this situation and Jenner's variolation with the vaccinia virus was immediately apparent to him and in honor of Jenner, Pasteur called his treatment **vaccination**.

In 1886, Theobold Smith (an American microbiologist) demonstrated that *heat killed* cultures of chicken cholera bacillus were also effective in protection from cholera. This demonstrated that the microorganisms **did not have to be viable** to induce the protection.

Pasteur proceeded to do the same with anthrax. Robert Koch had shown that the disease was due to a bacterium called *Bacillus anthracis*. He maintained the culture under adverse laboratory conditions (incubation at 42-43C) and in this manner, he produced the vaccine that was used for the famous demonstration at Pouilly-le-Fort.

On May 5, 1881 Pasteur vaccinated 24 sheep, 1 goat, and 6 cows with five drops of the living attenuated anthrax bacillus. On May 17, he inoculated all of the animals with a less attenuated strain. On May 31, all of the animals received **viable virulent** anthrax bacilli. Additionally, 24 more sheet, 1 goat and 3 cows received the virulent microorganism without the protection of the vaccination. On June 2, 1881 all of the non vaccinated animals had died while only two sheep of the protected group had succumbed. One of the sheep had died due to pregnancy complications.

A third means of virulence attenuation was soon found to involve the passage of the microorganism in an unnatural host. The most dramatic demonstration of a vaccine's effectiveness was with rabies. Isolating the virus from a fox, Pasteur passed the virus in an "unnatural host" the rabbit. By infecting one rabbit, allowing it to become ill, and then re-isolating the virus and injecting a new rabbit, Pasteur "selected" for variants of the virus that were less pathogenic for the fox. Pasteur dried the spinal cord taken from an infected rabbit and prepared a vaccine from it. To test

it however, he needed a human subject who was undoubtedly going to come down with rabies.

The first human trial was on July 6, 1885. A nine-year old lad named Joseph Meister had been severely bitten by a rabid dog two days earlier. His parents knew that he "was a goner" and were desperate for any ray of hope. They heard of Pasteur's work and traveled to Paris, France in hopes that Pasteur would "work a miracle". Pasteur injected the attenuated virus into young Joey and they waited. Well, you can guess the rest of the story. Joseph Meister, after receiving the immunization, survived rabies. This was the first known case of an individual being bit and surviving rabies. Joseph Meister was later to become the gate porter of the Pasteur Institute in Paris and served as guard to Pasteur's crypt. Within a year, over 350 people bitten by rabid animals had been treated with no fatalities.

Other events soon served to catapult immunology from the microbiology labs.

In 1888, Roux and Yersin discovered the diphtheria toxin. Two years later, in 1890, Von Behring and Kitasato demonstrated the presence of **anti-toxin** in the blood of individuals recovering from diphtheria. Von Behring was the first to use this antiserum in treating active disease. Forerunner to what we call 'serotherapy' today.

The ideas of circulating neutralizing anti-toxins predominated early immunological thought and the French and German schools dominated immunological research. At the turn of the century, immunology developed into two schools of thought.

1. Humoralists - immunity was due to *humoral* substances, i.e. antibodies. A Pioneer in this area was Paul Ehrlich. He proposed what was the most plausible humoral theory of antibody formation the 'side chain theory'. Emil Von Behring (worked at the Koch Institute in Germany) used serum to treat diseases. Germans were big proponents of **humoral immunity**.

2. Cellularists - immunity due to the existence of 'phagocytic' cells within our bodies. The pioneer here was Eli Metchnikoff and he became the strongest proponent of **cellular immunity** after observing water Daphnia phagocytose smaller materials and examining blood cells devour foreign bacteria in blood samples. Metchnikoff was allied with Louis Pasteur (he worked at the Pasteur Institute) and he had many a vitriolic fights with the Germans who were proponents of humoral immunity.

Today, we know that immunity is due to both of these facets. We will address humoral antibody in the form of immunoglobulins and we will talk about Cellularists

when we deal with T cell biology and cytotoxicity with regulation.

1903, Maurice Arthus, described the localizing allergic reaction called the Arthus response. In 1905, a Frenchman by the name of Von Pirquet shocked the world when he provided evidence that immune responses can be deleterious. He was studying serum sickness, a form of hypersensitivity or allergy.

- 1

930, American scientists Landsteiner and Kabat described detailed experiments of the specificity of the immune response by chemically altering antigens. In 1944-45, another American named Peter Medawar provided the immunological basis of transplantation immunology.

- 1

958, clonal selection theory as proposed by Sir McFarland Burnet and Neils Jerne

- 1

960, Porter and Edelman enzymatically digested antibodies and we learned about their chemical structure.

- 1

960, The first real demonstration of a cellular basis for humoral and cellular immunity. The terms T and B cells became vernacular.

There is, of course, a lot more immunology. The late 60's to early 70's have been referred to as the beginning of modern immunology. The molecular/genetic techniques of the 70's have revolutionized our understanding of how the immune system works.

Timeline of immunology:

- 1

549 - The earliest account of inoculation of smallpox (variolation) occurs in Wan Quan's (1499– 1582) *Douzhen Xinfa*

- 1

718 –smallpox vaccination in Ottoman Empire realized by west. Lady Mary Wortley Montagu, the wife of

the British ambassador to Constantinople, observed the positive effects of variolation on the native population

and had the technique performed on her own children.

- 1

796 – First demonstration of vaccination smallpox vaccination (Edward Jenner)

- 1

837 – Description of the role of microbes in putrefaction and fermentation (Theodore Schwann)

- 1

838 – Confirmation of the role of yeast in fermentation of sugar to alcohol (Charles Cagniard-Latour)

- 1

840 – Proposal of the germ theory of disease (Jakob Henle)

- 1

850

– Demonstration of the contagious nature of puerperal fever (childbed fever) (Ignaz Semmelweis)

- 1

857-1870 – Confirmation of the role of microbes in fermentation (Louis Pasteur)

- 1

862 – phagocytosis (Ernst Haeckel)

- 1

867 – Aseptic practice in surgery using carbolic acid (Joseph Lister)

- 1

876 – Demonstration that microbes can cause disease-anthrax (Robert Koch)

- 1

877 – Mast cells (Paul Ehrlich)

- 1

878 – Confirmation and popularization of the germ theory of disease (Louis Pasteur)

- 1

880 – 1881 -Theory that bacterial virulence could be attenuated by culture in vitro and used as vaccines.

Proposed that live attenuated microbes produced immunity by depleting host of vital

trace nutrients. Used to

make chicken cholera and anthrax "vaccines" (Louis Pasteur)

- 1

883 – 1905 – Cellular theory of immunity via phagocytosis by macrophages and microphages

(polymorhonuclear leukocytes) (Elie Metchnikoff)

- 1

885 – Introduction of concept of a "therapeutic vaccination". Report of a live "attenuated" vaccine for rabies

(Louis Pasteur).

- 1

888 – Identification of bacterial toxins (diphtheria bacillus) (Pierre Roux and Alexandre Yersin)

- 1

888 – Bactericidal action of blood (George Nuttall)

- 1

890 – Demonstration of antibody activity against diphtheria and tetanus toxins. Beginning of humoral theory

of immunity. (Emil von Behring) and (Kitasato Shibasaburō)

- 1

891 – Demonstration of cutaneous (delayed type) hypersensitivity (Robert Koch)

- 1

893 – Use of live bacteria and bacterial lysates to treat tumors-"Coley's Toxins" (William B. Coley)

- 1

894 – Bacteriolysis (Richard Pfeiffer)

- 1

896 – An antibacterial, heat-labile serum component (complement) is described (Jules Bordet)

- 1

900 – Antibody formation theory (Paul Ehrlich)

- 1

901 – blood groups (Karl Landsteiner)

- 1

902 – Immediate hypersensitivity anaphylaxis (Paul Portier) and (Charles Richet)

- 1

903 – Intermediate hypersensitivity, the "Arthus reaction" (Maurice Arthus)

- 1

903 – Opsonization

- 1

905 – "Serum sickness" allergy (Clemens von Pirquet and (Bela Schick)

- 1

909 – Paul Ehrlich proposes "immune surveillance" hypothesis of tumor recognition and eradication

- 1

911 – 2nd demonstration of filterable agent that caused tumors (Peyton Rous)

- 1

917 – hapten (Karl Landsteiner)

- 1

921 – Cutaneous allergic reactions (Otto Prausnitz and Heinz Küstner)

- 1

924 – Reticuloendothelial system

- 1

938 – Antigen-Antibody binding hypothesis (John Marrack)

- 1

940 – Identification of the Rh antigens (Karl Landsteiner and Alexander Weiner)

- 1

942 – Anaphylaxis (Karl Landsteiner and Merill Chase)

- 1

942 – Adjuvants (Jules Freund and Katherine McDermott)

- 1

944 – hypothesis of allograft rejection

- 1

945 – Coombs Test aka antiglobulin test (AGT)

- 1

946 – identification of mouse MHC (H2) by George Snell and Peter A. Gorer

- 1

948 – antibody production in plasma B cells

- 1

949 – growth of polio virus in tissue culture, neutralization with immune sera, and demonstration of

attenuation of neurovirulence with repetitive passage (John Enders) and (Thomas Weller) and (Frederick

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Robbins)

- 1

951 – vaccine against yellow fever

- 1

953 – Graft-versus-host disease

- 1

953 – Validation of immunological tolerance hypothesis

- 1

957 – Clonal selection theory (Frank Macfarlane Burnet)

- 1

957 – Discovery of interferon by Alick Isaacs and Jean Lindenmann[2]

- 1

958–1962 – Discovery of human leukocyte antigens (Jean Dausset and others)

- 1

959–1962 – Discovery of antibody structure (independently elucidated by Gerald Edelman and Rodney

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Porter)

- 1

959 – Discovery of lymphocyte circulation (James Gowans)

- 1

960 – Discovery of lymphocyte "blastogenic transformation" and proliferation in response to mitogenic

lectins-phytohemagglutinin (PHA) (Peter Nowell)

- 1

961-1962 Discovery of thymus involvement in cellular immunity (Jacques Miller)

- 1

961- Demonstration that glucocorticoids inhibit PHA-induced lymphocyte proliferation (Peter Nowell)

- 1

963 – Development of the plaque assay for the enumeration of antibody-forming cells in vitro by Niels

Jerne and Albert Nordin

- 1

963 Gell and Coombs classification of hypersensitivity

- 1

964-1968 T and B cell cooperation in immune response

- 1

965 – Discovery of lymphocyte mitogenic activity, "blastogenic factor" (Shinpei Kamakura) and (Louis

Lowenstein) (J. Gordon) and (L.D. MacLean)

- 1

965 – Discovery of "immune interferon" (gamma interferon) (E.F. Wheelock)

- 1

965 – Secretory immunoglobulins

- 1

967 – Identification of IgE as the reaginic antibody (Kimishige Ishizaka)

- 1

968 – Passenger leukocytes identified as significant immunogens in allograft rejection (William L.

Elkins and Ronald D. Guttmann)

- 1

969 – The lymphocyte cytolysis Cr51 release assay (Theodore Brunner) and (Jean-Charles Cerottini)

- 1

971 – Peter Perlmann and Eva Engvall at Stockholm University invented ELISA

- 1

972 – Structure of the antibody molecule

- 1

973 – Dendritic Cells first described by Ralph M. Steinman

- 1

974 - Immune Network Hypothesis (Niels Jerne)

- 1

974 – T-cell restriction to MHC (Rolf Zinkernagel and (Peter C. Doherty)

- 1

975 – Generation of monoclonal antibodies (Georges Köhler) and (César Milstein)[3]

- 1

975 - Discovery of Natural Killer cells (Rolf Kiessling, Eva Klein, Hans Wigzell)

- 1

976 – Identification of somatic recombination of immunoglobulin genes (Susumu Tonegawa)

- 1

980-1983 – Discovery and characterization of interleukins, 1 and 2 IL-1 IL-2 (Robert Gallo, Kendall A. Smith, Tadatsugu Taniguchi)

- 1

983 – Discovery of the T cell antigen receptor TCR (Ellis Reinherz) (Philippa Marrack) and (John K appler)[4] (James Allison)

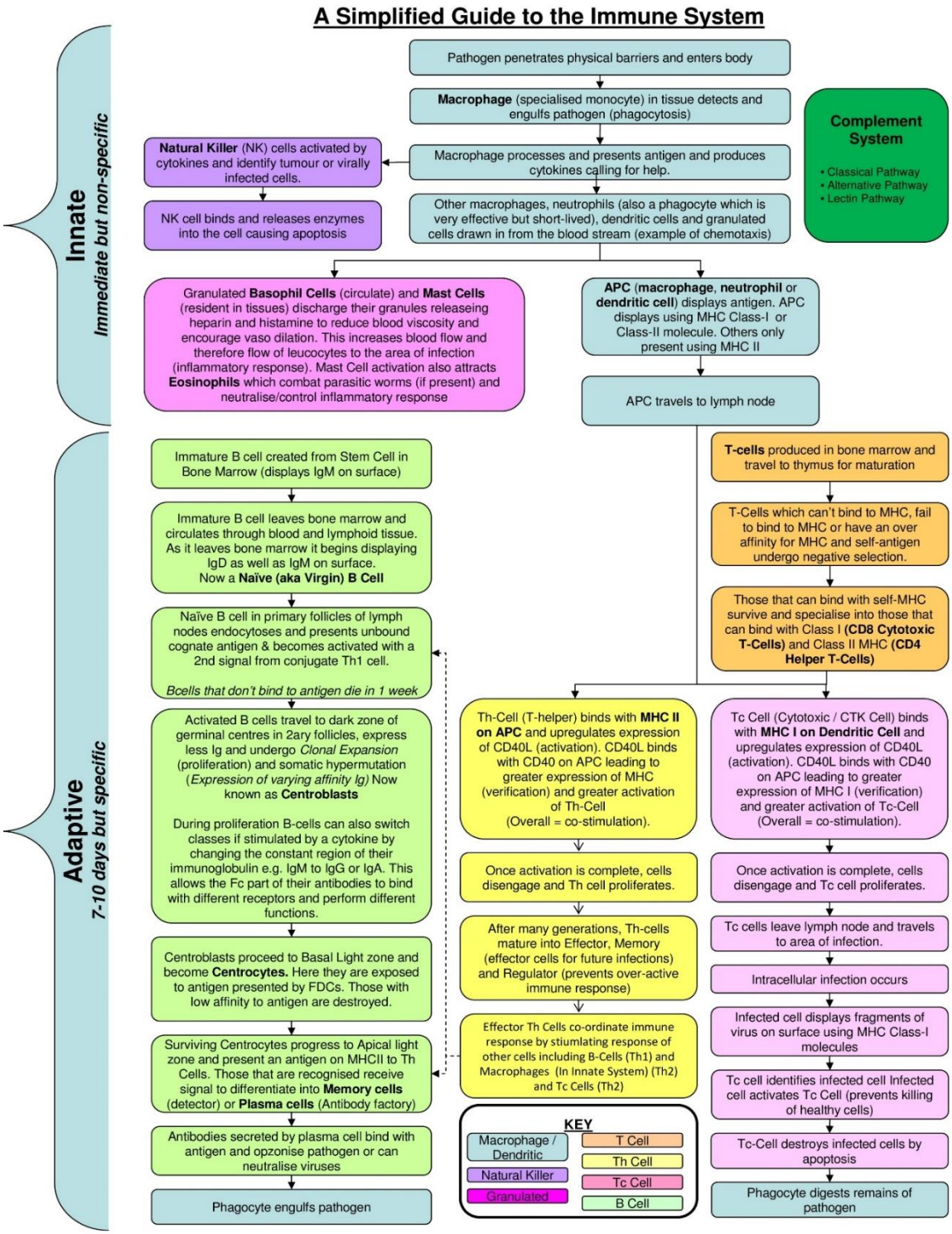
- 1

983 – Discovery of HIV (Luc Montagnier)

- 1985-1987 – Identification of genes for the T cell receptor
- 1986 – Hepatitis B vaccine produced by genetic engineering
- 1986 – Th1 vs Th2 model of T helper cell function (Timothy Mosmann)
- 1988 – Discovery of biochemical initiators of T-cell activation: CD4- and CD8-p56lck complexes (Christopher E. Rudd)
- 1990 – Gene therapy for SCID
- 1991 – Role of peptide for MHC Class II structure (Scheherazade Sadegh-Nasseri & Ronald N. Germain)
- 1992- Discovery of transitional B cells (David Allman & Michael Cancro) [5][6]
- 1994 – 'Danger' model of immunological tolerance (Polly Matzinger)
- 1995 – James P. Allison describes the function of CTLA-4
- 1995 – Regulatory T cells (Shimon Sakaguchi)
- 1995 – First Dendritic cell vaccine trial reported by Mukherji et al.
- 1996 - 1998 – Identification of Toll-like receptors
- 2000 - Discovery of M1 and M2 macrophage subsets (Charles Mills)[7]
- 2001 – Discovery of FOXP3 – the gene directing regulatory T cell development
- 2005 – Development of human papillomavirus vaccine (Ian Frazer)

- 2010 – First immune checkpoint inhibitor, ipilimumab (anti-CTLA-4), is approved by the FDA for treatment of stage IV melanoma
- 2011 – Carl June reports first successful use of CAR T-cells for the treatment of CD19+ malignancies
- 2014 – A second class of immune checkpoint inhibitor (anti-PD-1) is approved by the FDA for the treatment of melanoma. Two different drugs, pembrolizumab and nivolumab are approved within months of each other.
- 2016 – Matthew M. Halpert first characterizes role of dendritic cell CTLA-4 in Th-1 immunity

IMMUNITY



INNATE IMMUNITY : Every day we are alive, humans encounter potentially harmful disease causing organisms, or "pathogens", like bacteria or viruses. Yet most of us are still able

to function properly and live life without constantly being sick. That's because the human body requires a multilayered immune system to keep it running smoothly. The two main classes of the immune system are the innate immune system and the adaptive immune system, or "acquired immunity". In this article, we'll discuss the first line of defense: the innate immune system.

Self vs. Non-self: How does the body know?

In order to be effective, the immune system needs to be able to identify which particles are foreign, and which are a part of your body

Self refers to particles, such as proteins and other molecules, that are a part of, or made by, your body. They can be found circulating in your blood or attached to different tissues. Something that is self should not be targeted and destroyed by the immune system. The non-reactivity of the immune system to self particles is called tolerance.

Non-self refers to particles that are not made by your body, and are recognized as potentially harmful. These are sometimes called *foreign bodies*. Non-self particles or bodies can be bacteria, viruses, parasites, pollen, dust, and toxic chemicals. The non-self particles and foreign bodies that are infectious or pathogenic, like bacteria, viruses, and parasites, make proteins called antigens that allow the human body to know that they intend to cause damage.

Antigens are anything that causes an immune response. Antigens can be entire pathogens, like bacteria, viruses, fungi, and parasites, or smaller proteins that pathogens express. Antigens are like a name tag for each pathogen that announce the pathogens' presence to your immune system. Some pathogens are general, whereas others are very specific. A general antigen would announce "I'm dangerous", whereas a specific antigen would announce "I'm a bacteria that will cause an infection in your gastrointestinal tract" or "I'm the influenza vir

Cytokines are molecules that are used for cell signaling, or cell-to-cell communication. Cytokines are similar to chemokines, wherein they can be used to communicate with neighboring or distant cells about initiating an immune response. Cytokines are also used to trigger cell trafficking, or movement, to a specific area of the body.

Chemokines are a type of cytokines that are released by infected cells. Infected host cells release chemokines in order to initiate an immune response, and to warn neighboring cells of the threat.

Innate Immune System

The innate immune system is made of defenses against infection that can be activated immediately once a pathogen attacks. The innate immune system is essentially made up of barriers that aim to keep viruses, bacteria, parasites, and other

foreign particles out of your body or limit their ability to spread and move throughout the body. The innate immune system includes:

Physical Barriers

such as skin, the gastrointestinal tract, the respiratory tract, the nasopharynx, cilia, eyelashes and other body hair.

*Defense Mechanisms*such as secretions, mucous, bile, gastric acid, saliva, tears, and sweat.

General Immune Responses

such as inflammation, complement, and non-specific cellular responses. The inflammatory response actively brings immune cells to the site of an infection by increasing blood flow to the area. Complement is an immune response that marks pathogens for destruction and makes holes in the cell membrane of the pathogen.

The innate immune system is always general, or *nonspecific*, meaning anything that is identified as foreign or *non-self* is a target for the innate immune response. The innate immune system is activated by the presence of antigens and their chemical properties.

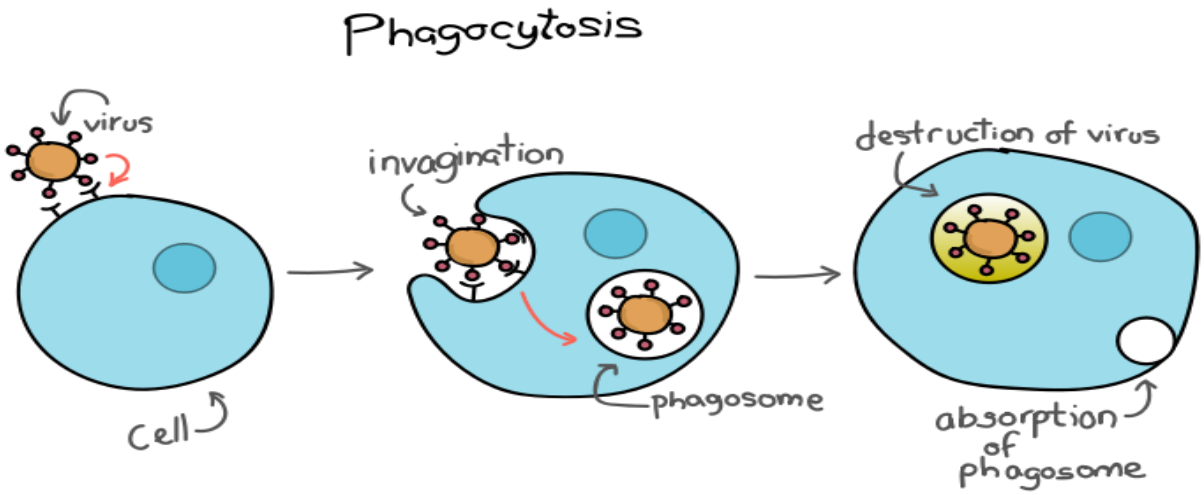
Cells of the Innate Immune System

There are many types of white blood cells, or *leukocytes*, that work to defend and protect the human body. In order to patrol the entire body, leukocytes travel by way of the circulatory system.

The following cells are leukocytes of the innate immune system:

- *P*

hagocytes, or *Phagocytic cells*: Phagocyte means “eating cell”, which describes what role phagocytes play in the immune response. Phagocytes circulate throughout the body, looking for potential threats, like bacteria and viruses, to engulf and destroy. You can think of phagocytes as security guards on patrol.



Phagocytosis diagram explains how phagocytes know what to engulf, and how phagocytosis works.

- *M*

acrophages: Macrophages, commonly abbreviated as "Mφ", are efficient phagocytic cells that can leave the circulatory system by moving across the walls of capillary vessels. The ability to roam outside of the circulatory system is important, because it allows macrophages to hunt pathogens with less limits. Macrophages can also release cytokines in order to signal and recruit other cells to an area with pathogens.

- *M*

ast cells: Mast cells are found in mucous membranes and connective tissues, and are important for wound healing and defense against pathogens via the inflammatory response. When mast cells are activated, they release cytokines and granules that contain chemical molecules to create an *inflammatory cascade*. Mediators, such as histamine, cause blood vessels to dilate, increasing blood flow and cell trafficking to the area of infection. The cytokines released during this process act as a messenger service, alerting other immune cells, like neutrophils and macrophages, to make their way to the area of infection, or to be on alert for circulating threats.

- *N*

eutrophils: Neutrophils are phagocytic cells that are also classified as *granulocytes* because they contain granules in their cytoplasm. These granules are very toxic to bacteria and fungi, and cause them to stop proliferating or die on contact.

The bone marrow of an average healthy adult makes approximately 100 billion new neutrophils per day. Neutrophils are typically the first cells to arrive at the site of an infection because there are so many of them in circulation at any given time.

Eosinophils: Eosinophils are granulocytes target multicellular parasites. Eosinophils secrete a range of highly toxic proteins and free radicals that kill bacteria and parasites. The use of toxic proteins and free radicals also causes tissue damage during allergic reactions, so activation and toxin release by eosinophils is highly regulated to prevent any unnecessary tissue damage.

While eosinophils only make up 1-6% of the white blood cells, they are found in many locations, including the thymus, lower gastrointestinal tract, ovaries, uterus, spleen, and lymph nodes.

Basophils: Basophils are also granulocytes that attack multicellular parasites. Basophils release histamine, much like mast cells. The use of histamine makes basophils and mast cells key players in mounting an allergic response.

Natural Killer cells: Natural Killer cells (NK cells), do not attack pathogens directly. Instead, natural killer cells destroy infected host cells in order to stop the spread of an infection. Infected or compromised host cells can signal natural kill cells for destruction through the expression of specific receptors and antigen presentation.

Dendritic cells: Dendritic cells are antigen-presenting cells that are located in tissues, and can contact external environments through the skin, the inner mucosal lining of the nose, lungs, stomach, and intestines. Since dendritic cells are located in tissues that are common points for initial infection, they can identify threats and act as messengers for the rest of the immune system by antigen presentation. Dendritic cells also act as bridge between the innate immune system and the adaptive immune system.

The Complement System

The complement system (also called the *complement cascade*) is a mechanism that *complements* other aspects of the immune response. Typically, the complement system acts as a part of the innate immune system, but it can work with the adaptive immune system if necessary.

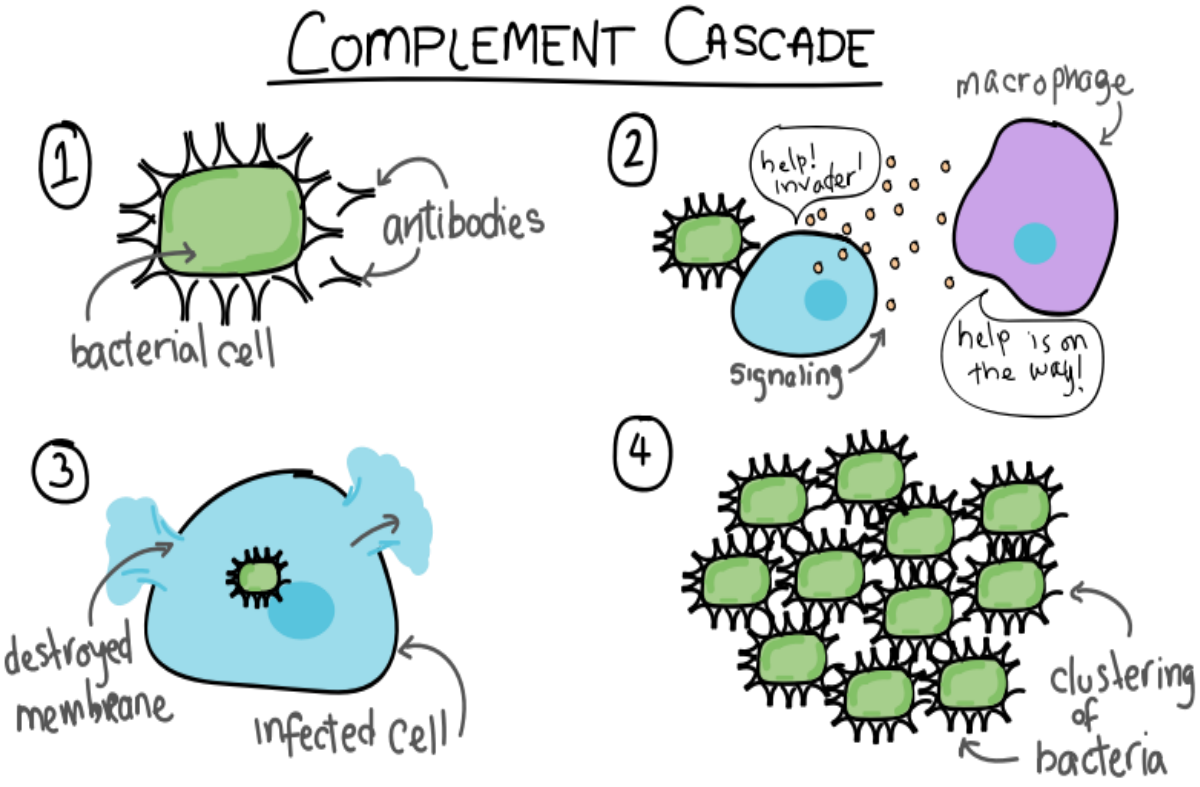
The complement system is made of a variety of proteins that, when inactive, circulate in the blood. When activated, these proteins come together to initiate the complement cascade, which starts the following steps:

Opsonization: Opsonization is a process in which foreign particles are marked for phagocytosis. All of the pathways require an antigen to signal that there is a threat present. Opsonization tags infected cells and identifies circulating pathogens expressing the same antigens.

Chemotaxis: Chemotaxis is the attraction and movement of macrophages to a chemical signal. Chemotaxis uses cytokines and chemokines to attract macrophages and neutrophils to the site of infection, ensuring that pathogens in the area will be destroyed. By bringing immune cells to an area with identified pathogens, it improves the likelihood that the threats will be destroyed and the infection will be treated.

Cell Lysis: Lysis is the breaking down or destruction of the membrane of a cell. The proteins of the complement system puncture the membranes of foreign cells, destroying the integrity of the pathogen. Destroying the membrane of foreign cells or pathogens weakens their ability to proliferate, and helps to stop the spread of infection.

Agglutination: Agglutination uses antibodies to cluster and bind pathogens together, much like a cowboy rounds up his cattle. By bringing as many pathogens together in the same area, the cells of the immune system can mount an attack and weaken the infection. Other innate immune system cells continue to circulate throughout the body in order to track down any other pathogens that have not been clustered and bound for destruction.



Complement cascade diagram

The steps of the complement cascade facilitate the search for and removal of antigens by placing them in large clumps, making it easier for other aspects of the immune system to do their jobs. Remember that the complement system is a supplemental cascade of proteins that assists, or "complements" the other aspects of the innate immune system.

The innate immune system works to fight off pathogens before they can start an active infection. For some cases, the innate immune response is not enough, or the pathogen is able to exploit the innate immune response for a way into the host cells. In such situations, the innate immune system works with the adaptive immune system to reduce the severity of infection, and to fight off any additional invaders while the adaptive immune system is busy destroying the initial infection.

Adaptive Immunity

Have you ever wondered how your recovery time for the common cold, the flu, or small infections seems to get shorter after you've been exposed and successfully recovered the first time? The adaptive immune system, also called *acquired immunity*, uses specific antigens to strategically mount an immune response. Unlike the innate immune system, which attacks only based on the identification of general threats, the adaptive immunity is activated by exposure to pathogens, and uses an immunological memory to learn about the threat and enhance the immune response accordingly. The adaptive immune response is much slower to respond to threats and infections than the innate immune response, which is primed and ready to fight at all times.

Cells of the adaptive immune system

Unlike the innate immune system, the adaptive immune system relies on fewer types of cells to carry out its tasks: *B cells* and *T cells*.

Both B cells and T cells are lymphocytes that are derived from specific types of stem cells, called multipotent hematopoietic stem cells, in the bone marrow. After they are made in the bone marrow, they need to mature and become activated. Each type of cell follows different paths to their final, mature forms.

B cells

After formation and maturation in the bone marrow (hence the name "B cell"), the naive *B cells* move into the lymphatic system to circulate throughout the body. In the lymphatic system, naive B cells encounter an antigen, which starts the maturation process for the B cell. B cells each have one of millions of distinctive surface antigen-specific receptors that are inherent to

the organism's DNA. For example, naive B cells express antibodies on their cell surface, which can also be called *membrane-bound antibodies*.

When a naive B cell encounters an antigen that fits or matches its membrane-bound antibody, it quickly divides in order to become either a *memory B cell* or an *effector B cell*, which is also called a *plasma cell*. Antibodies can bind to antigens directly.

The antigen must effectively bind with a naive B cell's membrane-bound antibody in order to set off *differentiation*, or the process of becoming one of the new forms of a B cell.

Memory B cells express the same membrane-bound antibody as the original naive B cell, or the "parent B cell". Plasma B cells produce the same antibody as the parent B cell, but they aren't membrane bound. Instead, plasma B cells can secrete antibodies. Secreted antibodies work to identify free pathogens that are circulating throughout the body. When the naive B cell divides and differentiates, both plasma cells and memory B cells are made.

B cells also express a specialized receptor, called the *B cell receptor (BCR)*. B cell receptors assist with antigen binding, as well as internalization and processing of the antigen. B cell receptors also play an important role in signaling pathways. After the antigen is internalized and processed, the B cell can initiate signaling pathways, such as cytokine release, 7 to communicate with other cells of the immune system. For more information on cell signalling, check out this article on cell-to-cell communication.

T cells

Once formed in the bone marrow, *T progenitor cells* migrate to the thymus (hence the name "T cell") to mature and become T cells. While in the thymus, the developing T cells start to express *T cell receptors (TCRs)* and other receptors called *CD4* and *CD8* receptors. All T cells express T cell receptors, and either CD4 or CD8, not both. So, some T cells will express CD4, and others will express CD8.

Unlike antibodies, which can bind to antigens directly, T cell receptors can only recognize antigens that are bound to certain receptor molecules, called *Major Histocompatibility Complex class 1 (MHCI)* and *class 2 (MHCII)*. These MHC molecules are membrane-bound surface receptors on *antigen-presenting cells*, like dendritic cells and macrophages. CD4 and CD8 play a role in T cell recognition and activation by binding to either MHCI or MHCII.

T cell receptors have to undergo a process called rearrangement, causing the nearly limitless recombination of a gene that expresses T cell receptors. The process of rearrangement allows for a lot of binding diversity. This diversity could potentially lead to accidental attacks against self cells and molecules because some rearrangement configurations can accidentally mimic a

person's self molecules and proteins. Mature T cells should recognize only foreign antigens combined with self-MHC molecules in order to mount an appropriate immune response.

In order to make sure T cells will perform properly once they have matured and have been released from the thymus, they undergo two selection processes:

1. *Positive* selection ensures MHC restriction by testing the ability of MHCI and MHCII to distinguish between self and nonself proteins. In order to pass the positive selection process, cells must be capable of binding only self-MHC molecules. If these cells bind nonself molecules instead of self-MHC molecules, they fail the positive selection process and are eliminated by apoptosis.
2. *Negative* selection tests for self tolerance. Negative selection tests the binding capabilities of CD4 and CD8 specifically. The ideal example of self tolerance is when a T cell will only bind to self-MHC molecules presenting a foreign antigen. If a T cell binds, via CD4 or CD8, a self-MHC molecule that isn't presenting an antigen, or a self-MHC molecule that presenting a self-antigen, it will fail negative selection and be eliminated by apoptosis.

These two selection processes are put into place to protect your own cells and tissues against your own immune response. Without these selection processes, autoimmune diseases would be much more common.

After positive and negative selection, we are left with three types of mature T cells: *Helper T cells*, *Cytotoxic T cells* and *T regulatory cells*.

- *Helper T* cells express CD4, and help with the activation of B cells, and other immune cells.

  Cytotoxic T cells express CD8, and are responsible for removing pathogens and infected host cells.

  T regulatory cells express CD4 and another receptor, called CD25. T regulatory cells help distinguish between self and nonself molecules, and by doing so, reduce the risk of autoimmune diseases.

Humoral vs. Cell Mediated Immunity

Immunity refers to the ability of your immune system to defend against infection and disease. There are two types of immunity that the adaptive immune system provides, and they are dependent on the functions of B and T cells, as described above.

Humoral immunity is immunity from serum antibodies produced by plasma cells. More specifically, someone who has never been exposed to a specific disease can gain humoral immunity through administration of antibodies from someone who has been exposed, and survived the same disease. "Humoral" refers to the bodily fluids where these free-floating serum antibodies bind to antigens and assist with elimination.

Cell-mediated immunity can be acquired through T cells from someone who is immune to the target disease or infection. "Cell-mediated" refers to the fact that the response is carried out by cytotoxic cells. Much like humoral immunity, someone who has not been exposed to a specific disease can gain cell-mediated immunity through the administration of cells from someone that has been exposed, and survived the same disease. The T cells act to activate other immune cells, while the cells assist with the elimination of pathogens and infected host cells.

Immunological memory

Because the adaptive immune system can learn and remember specific pathogens, it can provide long-lasting defense and protection against recurrent infections. When the adaptive immune system is exposed to a new threat, the specifics of the antigen are memorized so we are prevented from getting the disease again. The concept of immune memory is due to the body's ability to make antibodies against different pathogens.

A good example of immunological memory is shown in vaccinations. A vaccination against a virus can be made using either active, but weakened or attenuated virus, or using specific parts of the virus that are not active. Both attenuated whole virus and virus particles cannot actually cause an active infection. Instead, they mimic the presence of an active virus in order to cause an immune response, even though there are no real threats present. By getting a vaccination, you are exposing your body to the antigen required to produce antibodies specific to that virus, and acquire a memory of the virus, without experiencing illness.

Some breakdowns in the immunological memory system can lead to autoimmune diseases. Molecular mimicry of a self-antigen by an infectious pathogen, such as bacteria and viruses, may trigger autoimmune disease due to a cross-reactive immune response against the infection. One example of an organism that uses molecular mimicry to hide from immunological defenses is *Streptococcus* infection.

Innate Immunity vs. Adaptive Immunity: A summary

The following chart compares and summarizes all of the important parts of each immune system:

Attribute	Innate Immunity	Adaptive Immunity
Response Time	Fast: minutes or hours	Slow: days
Specificity	Only specific for molecules and molecular patterns associated with general pathogens or foreign particles	Highly specific! Can discriminate between pathogen vs. non-pathogen structures, and miniscule differences in molecular structures
Major Cell Types	Macrophages, Neutrophils, Natural Killer Cells, Dendritic Cells, Basophils, Eosinophils	T cells, B cells, and other antigen presenting cells
Key Components	Antimicrobial peptides and proteins, such as toxic granules	Antibodies
Self vs. Nonself Discrimination	Innate immunity is based on self vs. nonself discrimination, so it has to be perfect	Not as good as the innate immune system, but still pretty good at determining which is which. Problems in self vs. nonself discrimination result in autoimmune diseases
Immunological Memory	None	Memory used can lead to faster response to recurrent or subsequent infections
Diversity and Customization	Limited: Receptors used are standard and only recognize antigen patterns. No new receptors are made to adapt the immune response	Highly diverse: can be customized by genetic recombination to recognize epitopes and antigenic determinants.

Difference Between Humoral and Cell Mediated Immunity

Main Difference – Humoral Immunity vs Cell mediated immunity

Humoral immunity and cell mediated immunity are two types of adaptive immunity. Adaptive immunity generates an antigen-specific immune response. During adaptive immunity, the antigen is first recognized through receptors of the lymphocytes, and immune cell clones are produced to attack that particular antigen. Humoral immunity is triggered by B cells while

cell mediated immunity is triggered by T cells. The **main difference** between humoral and cell mediated immunity is that **antigen-specific antibodies are produced in humoral immunity whereas antibodies are not produced in cell mediated immunity**. Instead, T cells destroy the infected cells by inducing apoptosis.

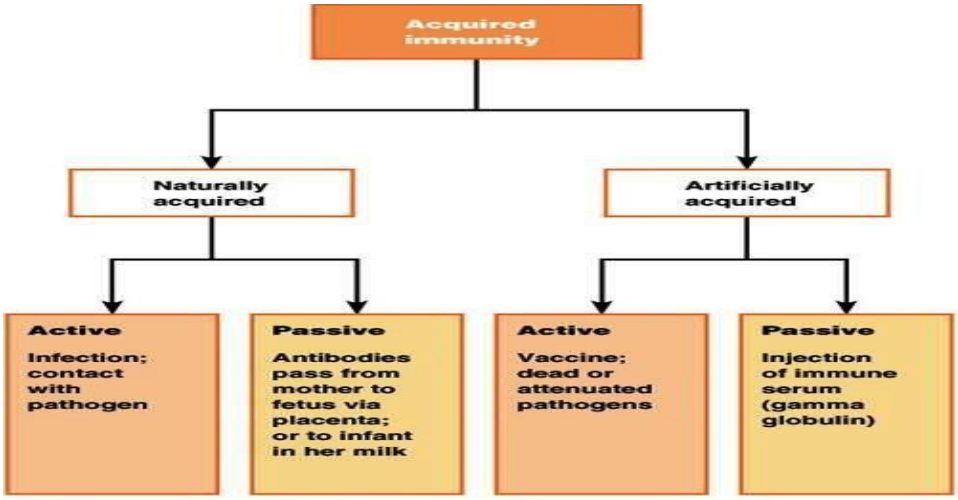
Primary and Secondary Immunity

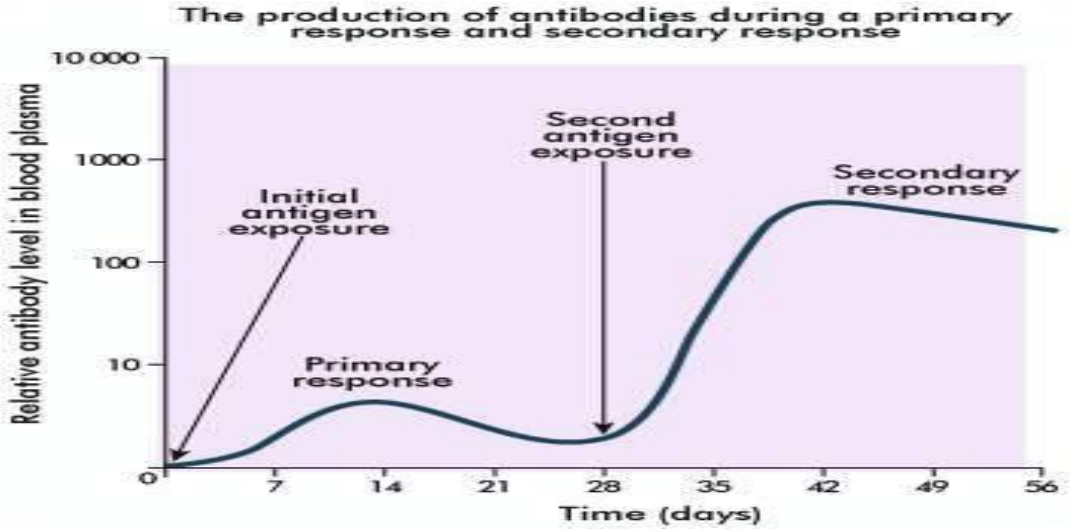
Primary Immunity – When first exposed to an antigen, the body usually takes several days to respond and build up a large supply of antibodies. The number of antibodies will peak and then begin to decline.

Secondary Immunity – The production of Memory B or T Cells allows the cell to recognize the antigen much quicker if it is introduced again so the body will often be able to destroy the invading antigen before its numbers become great enough to initiate symptoms. Memory B cells rapidly divide and develop into plasma cells and the antibody levels in the body rise quickly and reach greater numbers. Active immunity lasts as long as clones of memory B and memory T cells are present

Sources of Specific Immunity – resistance to a disease causing organism or harmful substance

- **Inborn Immunity** – Immunity for certain diseases is inherited
- **Acquired Immunity** – immunity can be acquired through infection or artificially by medical intervention





Natural

Immunity

exposure to causative agent or antigen is not deliberate and occurs in the course of everyday living as exposure to a disease causing pathogen or allergen

- **A**

ctive Exposure – you develop your own antibodies – Immunity is long lived

- **P**

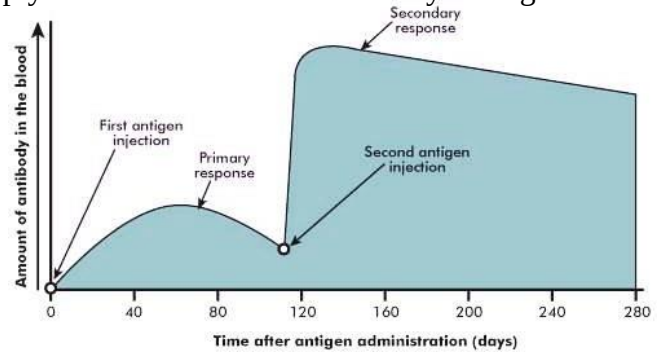
assive Exposure – you receive antibodies from another source as infants receiving antibodies from mother's milk. This immunity is short-lived

- ***A***

rtificial Immunity or ***Immunization*** – exposure to causative agent or antigen is deliberate

- **A**

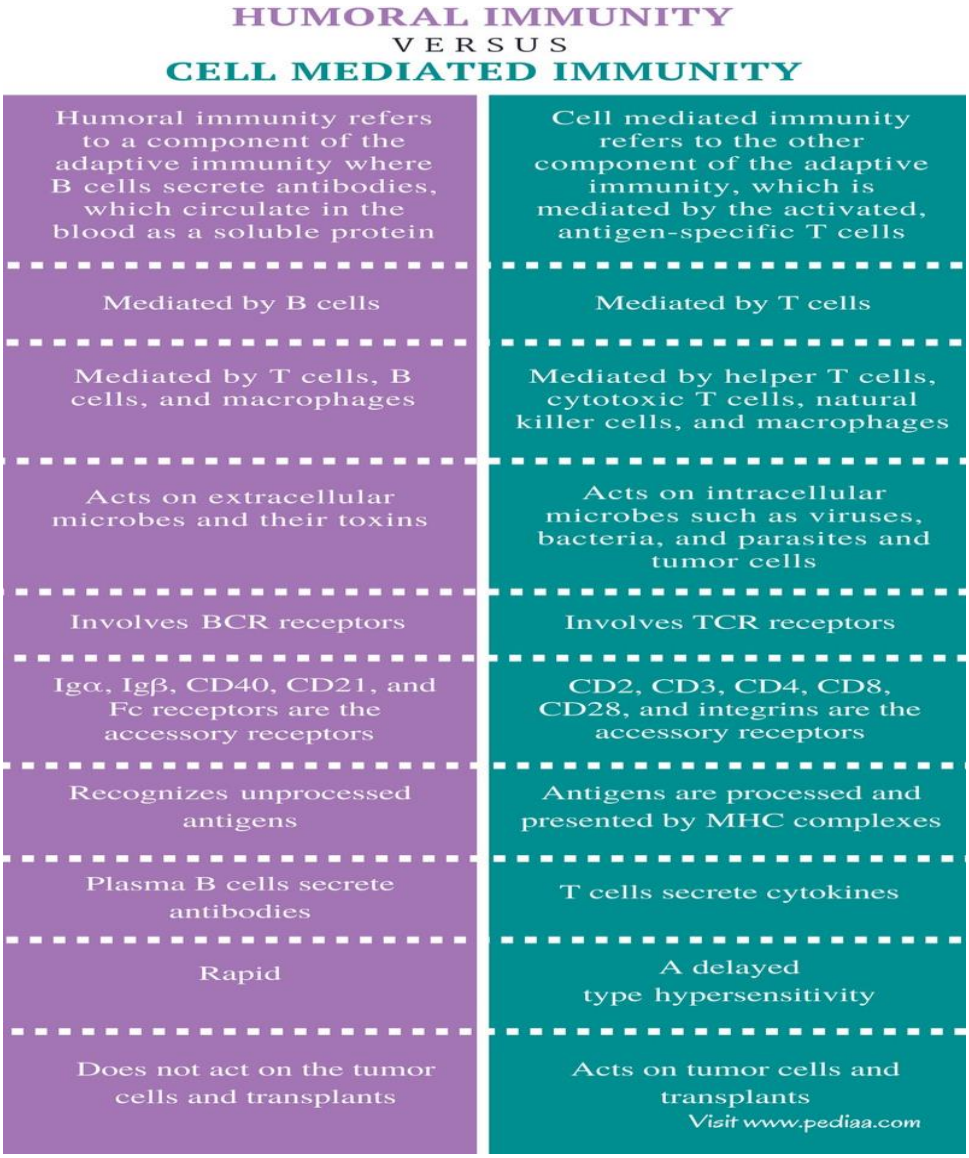
ctive Exposure – injection of causative agent that has been weakened or killed such as a **vaccine** and you develop your own antibodies - Immunity is long lived



Result of an initial immunization and a booster injection

- **Passive Exposure** - injection of protective **gamma globulin** serum containing antibodies that were developed by someone else's immune system - This immunity is short-

lived but immediate so it prevents full infection from developing in patients just exposed to serious agents when there is not time to develop active immunity from immunization



What is Humoral Immunity

Humoral immunity is the immunity generated by circulating antibodies. It is a component of adaptive immunity, which generates specific immune responses to a particular foreign material. The extracellular spaces of the body are

protected by humoral immunity. Most pathogens that invade the body multiply in the extracellular spaces. Intracellular pathogens move from one cell to another through the extracellular space. Therefore, extracellular space is an important place to destroy pathogens.

Antibodies are produced and secreted by plasma B cells. Typically, the activation of B cells occurs in T helper cells.

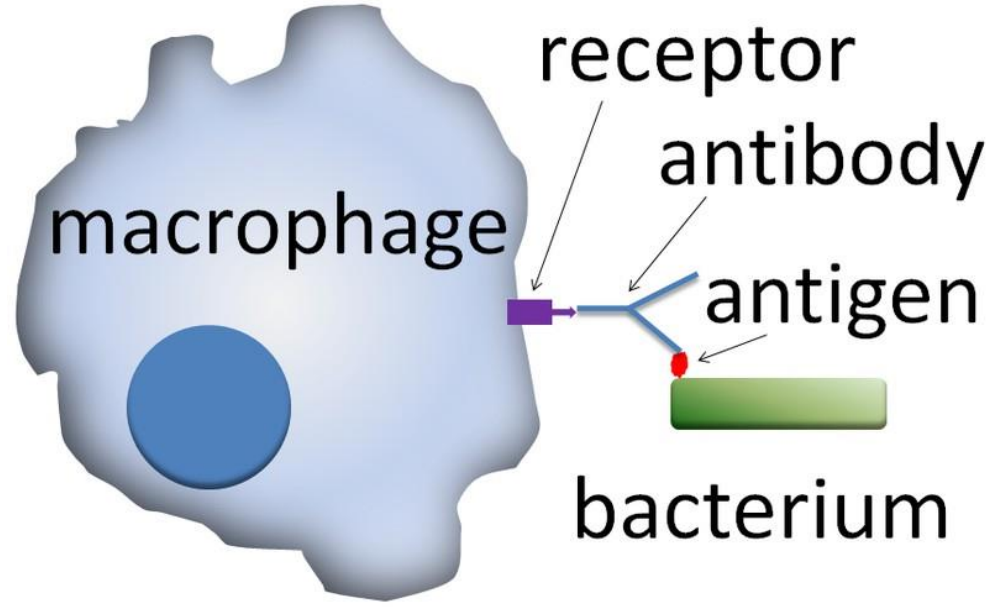


Figure 1: Opsonization

Antibodies destroy pathogens in three ways. They bind to the specific molecules on the surface of the pathogen, neutralizing the pathogen. This neutralization prevents the entering of the pathogen to the cells. It is also important to prevent bacterial toxins. The antibody-caught pathogens are subjected to **phagocytosis** by macrophages and other cells. This process is called **opsonization**. The binding of antibodies to the pathogens activates the complement system. The complement proteins bind to the antibody-bound pathogens and recruit phagocytic cells. The opsonization is shown in *figure 1*.

What is Cell Mediated Immunity

Cell mediated immunity is the immunity mediated by antigen-specific T cells. T cells are produced in the bone marrow and are matured in the thymus. After they enter the bloodstream, T cells occur can be found in the blood as well as in lymphoid tissue. The antigens should be presented on the surface of the antigen-presenting cells (APCs) along with the major histocompatibility complexes (MHC). Once T cells encounter an antigen, they proliferate and differentiate into armed effector cells. The cytotoxic T cells destroy the infected cells by inducing apoptosis. T helper cells stimulate plasma B cells to produce antibodies.

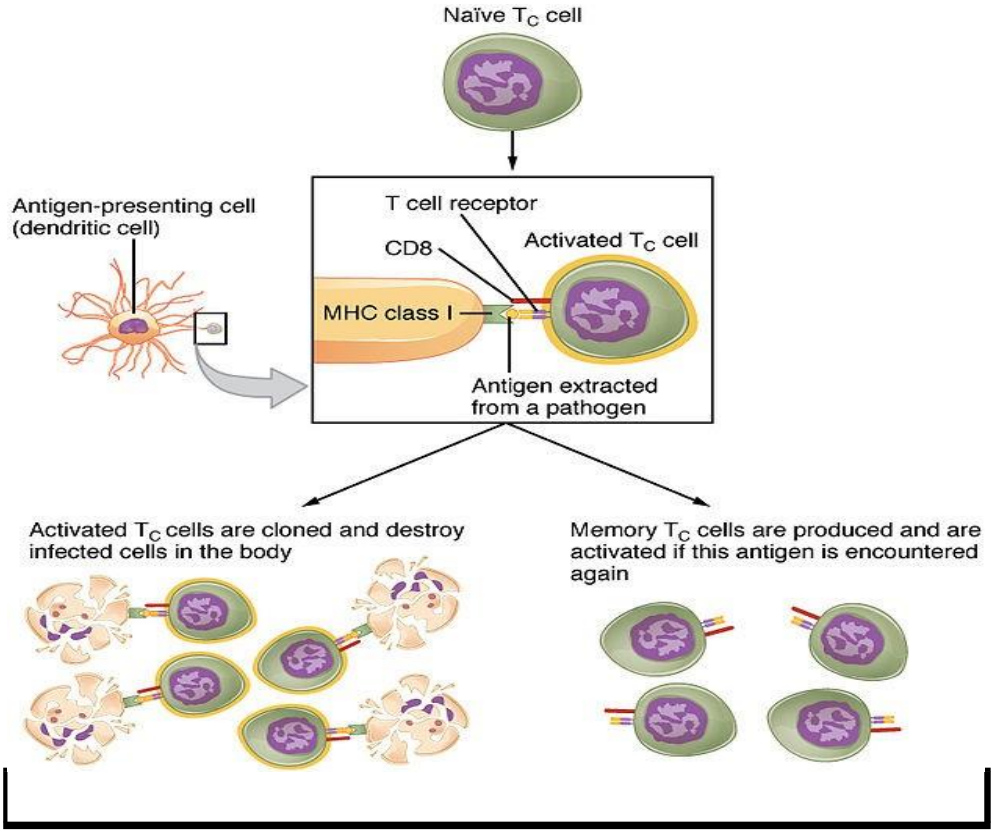


Figure 2: Cell Mediated Immunity

The IgG and IgM are the main two types of antibodies produced by T helper cells in response to plasma B cells. The memory T cells are differentiated T cells, but their action requires the activation by the specific antigen. The major characteristic feature of the cell mediated immunity is that it destroys intracellular pathogens. The cell mediated immunity is shown in *figure 2.*

Similarities Between Humoral Immunity and Cell Mediated Immunity

- Humoral immunity and cell mediated immunity are two types of adaptive immunity.
- Both humoral immunity and cell mediated immunity produce a specific immune response to a particular pathogen.

Difference Between Humoral and Cell Mediated Immunity

Definition

Humoral Immunity: Humoral immunity refers to a component of the adaptive immunity where B cells secrete antibodies, which circulate in the blood as a soluble protein.

ell Mediated Immunity: Cell mediated immunity refers to the other component of the adaptive immunity, which is mediated by the activated, antigen-specific T cells.

Main Cells

Humoral Immunity: The humoral immunity is mediated by B cells.

Cell Mediated Immunity: The cell mediated immunity is mediated by T cells.

Cell Types

Humoral Immunity: Humoral immunity is mediated by T cells, B cells, and macrophages.

Cell Mediated Immunity: Cell mediated immunity is mediated by helper T cells, cytotoxic T cells, natural killer cells, and macrophages.

Humoral Immunity: The humoral immunity acts on the extracellular microbes and their toxins.

Cell Mediated Immunity: The cell mediated immunity acts on intracellular microbes such as viruses, bacteria, and parasites and tumor cells.

Receptors

Humoral Immunity: The BCR receptors are involved in the humoral immunity.

Cell Mediated Immunity: The TCR receptors are involved in the cell mediated immunity.

Accessory Surface Molecules

Humoral Immunity: The Igα, Igβ, CD40, CD21, and Fc receptors are the accessory receptors of the humoral immunity.

Cell Mediated Immunity: The CD2, CD3, CD4, CD8, CD28, and integrins are the accessory receptors of the cell mediated immunity.

Role of MHC Molecules

Humoral Immunity: The unprocessed antigens are recognized by the humoral immunity.

Cell Mediated Immunity: The antigens are processed and presented by MHC complexes in the cell mediated immunity.

Secretion

Humoral Immunity: The plasma B cells secrete antibodies in the humoral immunity.

Cell Mediated Immunity: The T cells secrete cytokines.

Onset

Humoral Immunity: The humoral immune response is rapid.

Cell Mediated Immunity: The cell-mediated immune response is a delayed type of hypersensitivity.

Tumor Cells and Transplants

Humoral Immunity: The humoral immunity does not act on the tumor cells and transplants.

Cell Mediated Immunity: The cell mediated immunity acts on tumor cells and transplants.

IMMUNE SYSTEM

The **body's defense** against:

- disease causing organisms or infectious agents
- malfunctioning cells or abnormal body cells as cancer
- foreign cells or particles

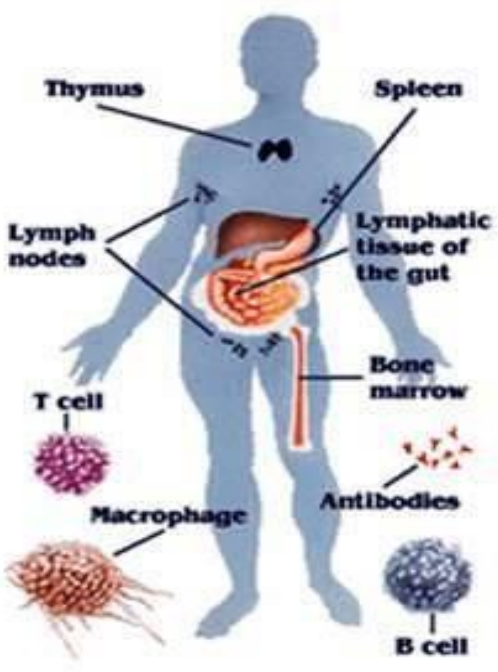
Basic Immunity

- Depends on the ability of the immune system to distinguish between ***self*** and ***non-self*** molecules
- ***Self*** molecules are those components of an organism's body that can be distinguished from foreign substances by the immune system
  - ***Autoimmunity*** is an immune reaction against self molecules (causes various diseases)
- ***Non-self*** molecules are those recognized as foreign molecules
  - One class of non-self molecules are called **antigens** (short for *anti*body *gen*erators) and are defined as substances that bind to specific immune receptors and elicit an immune response
  - Immune System Components:
  - **specific cells** - lymphocytes, macrophages, etc., originate from precursor cells in the bone marrow and patrol tissues by circulating in either the blood or lymphatics, migrating into connective tissue or collecting in immune organs

- **lymphatic organs**- thymus, spleen, tonsils, lymph nodes
- **diffuse lymphatic tissue** -collections of lymphocytes and other immune cells dispersed in the lining of the digestive and respiratory tracts and in the skin

Organs of the Lymphatic System Aid Immunity

Lymph Nodes



- Small (1- 25 mm) round structures found at points along lymphatic vessels that have fibrous connective tissue capsule with incoming and outgoing lymphatic vessels
- Each nodule contains sinus filled with lymphocytes and macrophages
- They occur in regions: auxiliary nodes in armpits and inguinal nodes in groin
- Occur singly or in groups of nodules:
  - **Tonsils** are located in back of mouth on either side
  - **Adenoids** on posterior wall above border of soft palate
  - **Peyer's patches** found within intestinal wall

Spleen

- Located in upper left ab dominal cavity just beneath diaphragm. Structure similar to lymph node; outer connective tissue divides organ into lobules with sinuses filled with blood
- Blood vessels of spleen can expand so spleen functions as blood reservoir making blood available in times of low pressure or oxygen need
- ***Red pulp*** containing RBCs, lymphocytes, and macrophages; functions to remove bacteria and worn-out red blood cells
- ***White pulp*** contains mostly lymphocytes
- Both help to purify the blood

Thymus

- Located along trachea behind sternum in upper thorax

- Larger in children; disappears in old age
- Divided into lobules where T lymphocytes mature
- Interior (medulla) of lobule secretes *thymosin* thought to aid T cells to mature

Red Bone Marrow

- Site of origin of all types of blood cells
- Five types of white blood cells (WBCs) function in immunity
- Stem cells continuously divide to produce cells that differentiate into various blood cells
- Most bones of children have red blood marrow
- In adult, red marrow is found in the skull, sternum, ribs, clavicle, spinal column, femur, and humerus
- Red blood marrow has network of connective tissue where reticular cells produce reticular fibers; these plus stem cells fill sinuses; differentiated blood cells enter bloodstream at these sinuses

Immune tissue associated with various organs:

GALT—***gut-associated*** lymphatic tissue; comprised of lymphoid tissue (lymph nodules) in the intestinal wall containing lymphocytes, plasma cells and macrophages.

- The digestive tract is a very important part of the immune system and the intestine possesses the largest mass of lymphoid tissue in the body.

Lymphoid tissue in the gut comprises the following:

- ***Tonsils*** (Waldeyer's ring)
- ***Adenoids*** (Pharyngeal tonsils)
- ***Peyer's patches* –** lymphoid follicles in wall of small intestine
- Lymphoid aggregates in the appendix and large intestine
- Lymphoid tissue accumulating with age in the stomach
- Small lymphoid aggregates in the esophagus
- Diffusely distributed lymphoid cells and plasma cells in lining of the gut

MALT—***mucosa-associated*** lymphatic tissue; lymphoid tissue associated with the mucosa of the female reproductive tract, respiratory tract, etc.

SALT—***skin-associated*** lymphatic tissue; lymphatic tissue associated with the dermis of the skin.

Plan of Protection – ***Immunity*** is the ability to defend against infectious agents, foreign cells and abnormal cells eg. cancerous cells

- **1st Line of defense** – Block entry
- **2nd Line of Defense** – Fight Local Infections
- **3rd Line of Defense** – Combat Major Infections

Nonspecific and Specific Defense Systems - work together to coordinate their responses

***Nonspecific (Innate) Response* - responds quickly, fights all invaders and consists of:**

- **First line of defense** – intact skin and mucosae and secretions of skin and mucous membranes prevent entry of microorganisms
- **Second line of defense** – phagocytic white blood cells, antimicrobial proteins, and other cells
- Inflammatory response process is key
- Inhibit invaders from spreading throughout the body

***Specific Response (Adaptive) Response* - takes longer to react, works on specific types of invaders which it identifies and targets for destruction**

- **Third line of defense** – mounts attack against particular foreign substances
- Lymphocytes and Antibodies
- Works in conjunction with the nonspecific or innate system

Nonspecific (Innate) Response – fight all invaders

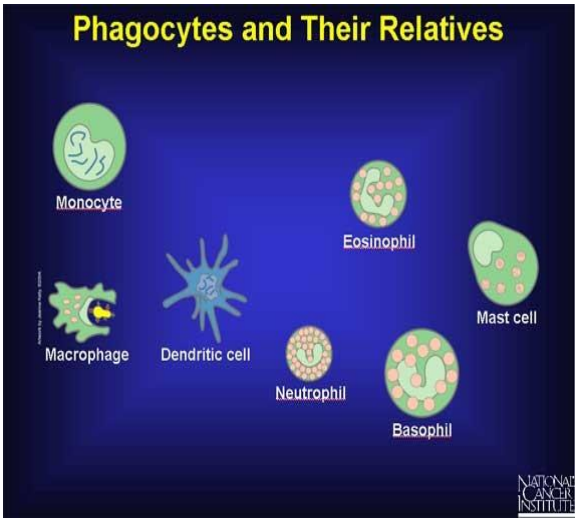
First line of defense – Non specific barriers to block entry

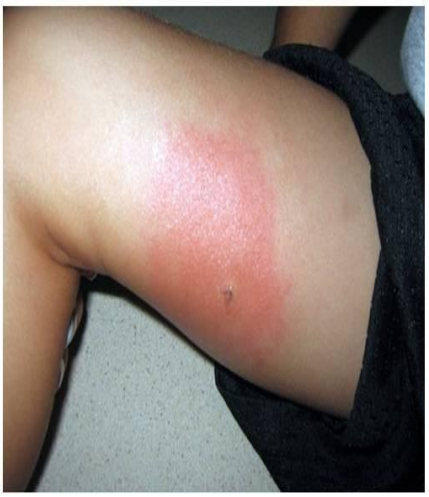
- Skin provides an impervious barrier – physical or mechanical barrier
- Mucous membranes line the entrances of the body and produce mucus which traps foreign particles and directs them out of the body – physical or mechanical barrier
- Nasal hairs trap dirt and dust while microscopic cilia line some mucous membranes helping to trap foreign particles
- Gastric juice, vaginal secretions and urine are acidic fluids which provide protection
- Natural flora (harmless bacteria) in the intestine and vagina prevent pathogens from growing
- Tears, saliva and sweat possess some anti-bacterial properties
- Cerumen or ear wax protects the ear canal by trapping dirt and dust particles

Second line of defense – Fight local infection with Inflammation Process

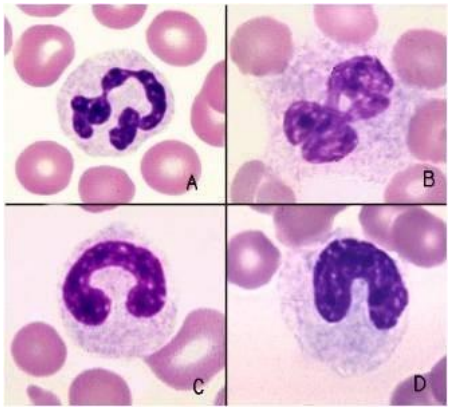
- Begins as soon as the first line of defense is violated
- The response is a non-specific, immediate, maximal response to the presence of any foreign organism or substance and involves no immunological memory

- **Phagocytosis** is an important feature of cellular innate immunity performed by cells called 'phagocytes' that engulf, or eat, pathogens or particles
- **Phagocytes** – types of immune cells involved in phagocytosis - Produced throughout life by the bone marrow
- **Scavengers** – remove dead cells and microorganisms
- **Complement proteins** activate other proteins in a domino fashion resulting in a cascade of reactions which attract phagocytes to the site of the invasion, bind to the surface of microbes to insure WBC's can phagocytize the microbe and produce holes in the bacterial cell walls and membranes
- The **Inflammation Process** releases ***histamines***causing redness, pain, swelling, and heat





Phagocytes and their Relatives

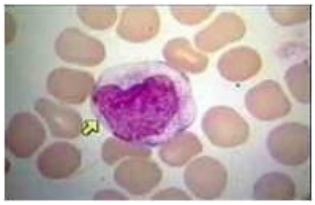


Neutrophils - kill bacteria

- 60% of WBCs
- 'Patrol tissues' as they squeeze out of the capillaries
- Large numbers are released during infections
- Short lived – die after digesting bacteria
- Dead neutrophils make up a large proportion of puss

Monocytes – are chief phagocytes found in the blood

- Made in bone marrow as ***monocytes*** and the circulate in the blood for 1-2 days before being called ***macrophages*** once they reach organs.



Macrophages - Found in the organs, not the blood

- Larger than neutrophils and long lived - involved in phagocytosis, release interferon and interleukin (which stimulates production of cells of the Specific Defense System)
- Macrophages also act as scavengers, ridding the body of worn-out cells and other debris by ingesting cellular debris, foreign material, bacteria and fungi
- Versatile cells that reside within tissues and produce a wide array of chemicals including enzymes, complement proteins, and regulatory factors such as interleukin 1
- ***Antigen-presenting cells*** that activate the adaptive immune system they display antigens from the pathogens to the lymphocytes.

Basophils – are capable of ingesting foreign particles and produce heparin and histamine and which induce inflammation, are often associated with asthma and allergies

Mast cells reside in connective tissues and mucous membranes, and regulate the inflammatory response.

They are most often associated with allergy and anaphylaxis: for example, they release histamine – this is why anti-histamines help allergic reactions

Dendritic cells are phagocytes in tissues that are in contact with the external environment

- Located mainly in the skin, nose, lungs, stomach, and intestines (are in no way connected to the nervous system)

Dendritic cells serve as a link between the innate and adaptive immune systems, as they present antigens to T cells, one of the key cell types of the adaptive immune system

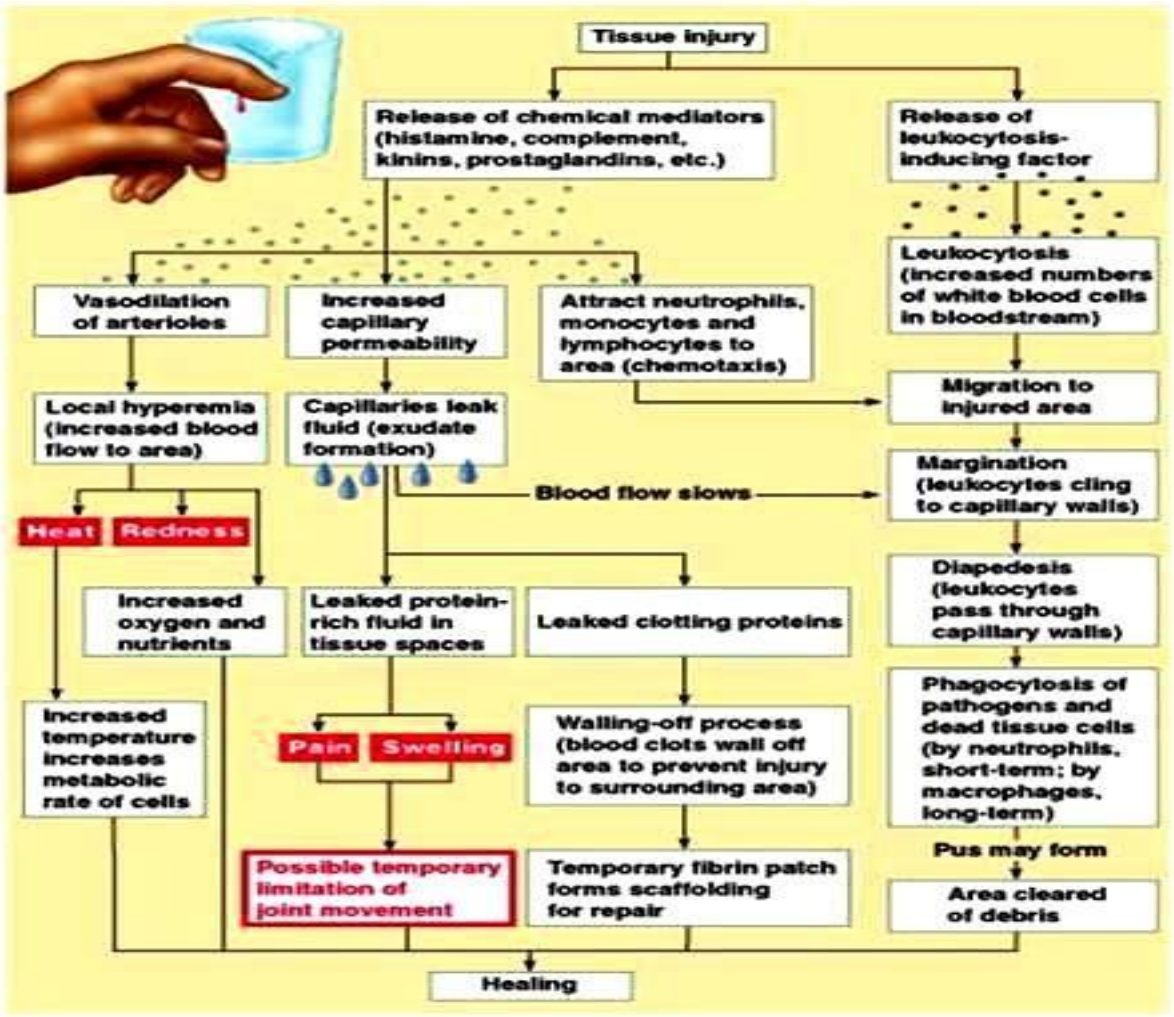
Eosinophils – weakly phagocytic of pathogens kill parasitic worms

NK cells (natural killer) - used to combat tumor cells or virus-infected cells

- A class of lymphocytes which attack and induce cells to kill themselves (self-induced apoptosis)

They complement both specific and nonspecific defenses

- May also attack some tumor cells
- Also secrete interferons, proteins produced by virus infected cells which binds to receptors of non-infected cells, causing these cells to produce a substance that will interfere with viral reproduction and activate macrophages and other immune cells



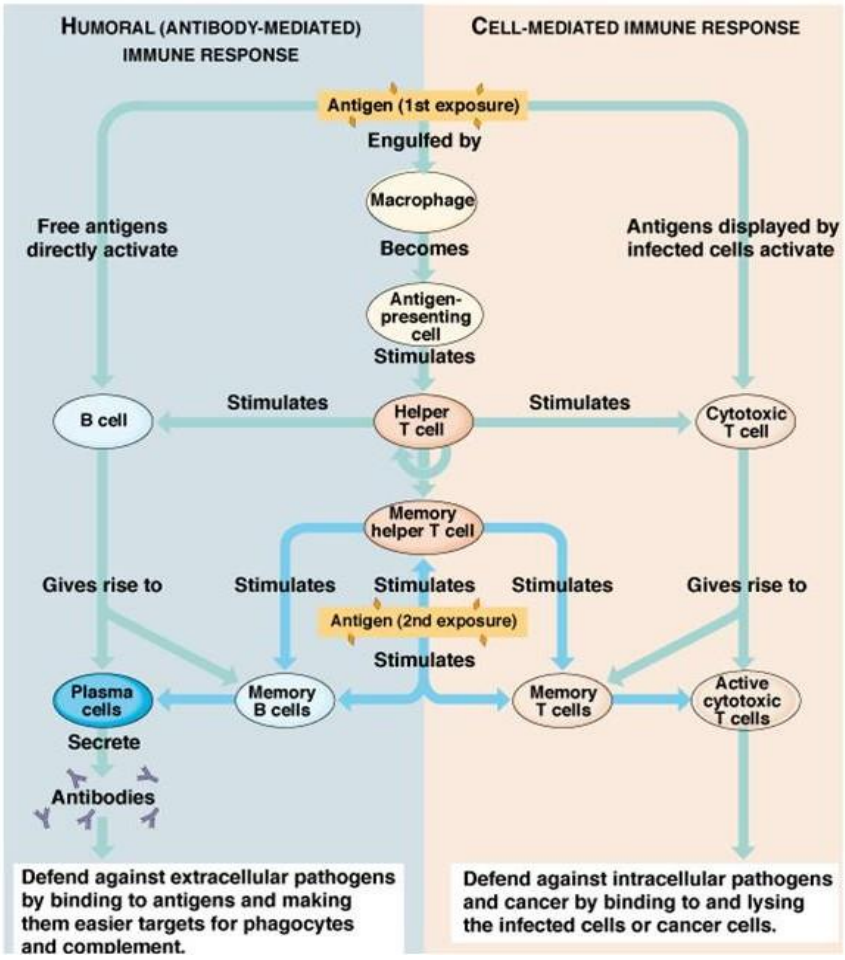
FLOW CHART OF INFLAMMATION PROCESS

Most infections never make it past the first and second level of defense.

Specific (Adaptive) Response – work on specific types of invaders which it identifies and targets for destruction - takes longer to react

- The response is directed at specific targets and is not restricted to initial site of invasion/infection
- Lag time occurs between exposure and maximal response
- The adaptive immune system allows for a stronger immune response as well as immunological memory, where each pathogen is "remembered" by its signature antigen
- **Antigens** are proteins or carbohydrate chain of a glycoprotein within a plasma membrane which the body recognizes as "nonself"
- The specific immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called **antigen presentation**
- Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells
- The ability to mount these tailored responses is maintained in the body by "**memory cells**"
- Should a pathogen infect the body more than once, these specific memory cells are used to ***quickly*** eliminate

Third line of defense – mounts attack against particular foreign substances antigens throughout the body



Components of the Specific Defense System

- Identify, destroy, remember
- **Cellular components** – B cells and T cells - lymphocytes which are white blood cells
- ***Humoral (antibody-mediated response)*** defends against extracellular pathogens by binding to antigens and making them easier targets for phagocytes and complement proteins
- ***Cell mediated immune response*** – defends against intracellular pathogens and cancer by binding to and lyzing the infected cells or cancer cells

Humoral or antibody-mediated response – termed anti-body mediated because B cells produce antibodies and Humoral because antibodies are released into the bloodstream

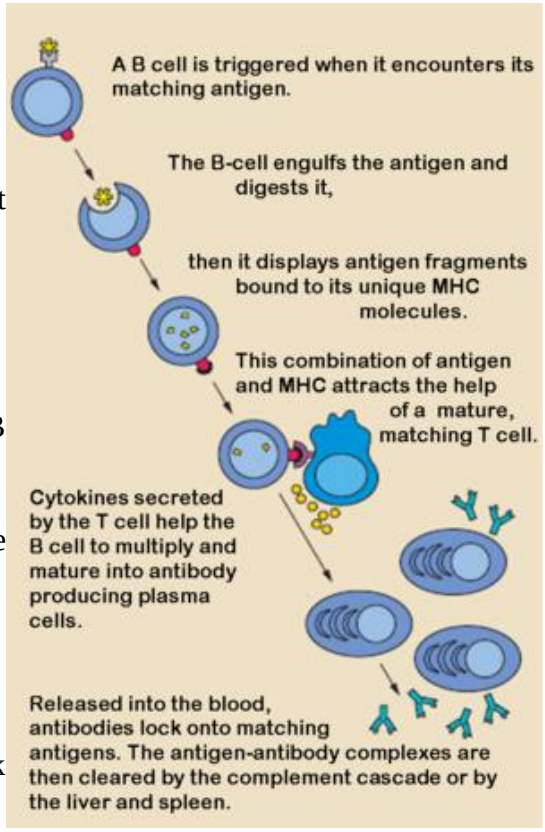
B cells - are produced and mature in the bone marrow – they possess a protein on the B cells outer surface known as the B cell receptor (BCR) which allows them to bind to a specific antigen

Plasma B cells also known as plasma cells, plasmocytes, and effector B cells– they produce antibodies

Memory B cells – ready for the next invasion

- B cell comes into contact with antigen on microbe, it attaches to the antigen and becomes an antigen-presenting
- B cell with antigen-MHC complex
- Helper T cell that binds to the complex
- Helper T secretes interleukin that stimulates mitosis in B cells so they multiply
- Some B cells mature into **plasma cells** and other become **memory cells**
- The **plasma cells** produce **antibodies** also called **immunoglobins** – proteins which attach to the antigens
- Antibodies can clump microbes for destruction, mark microbes

for destruction by phagocytes, activate complement proteins that rupture/lyse microbe cell membranes or infected host cells

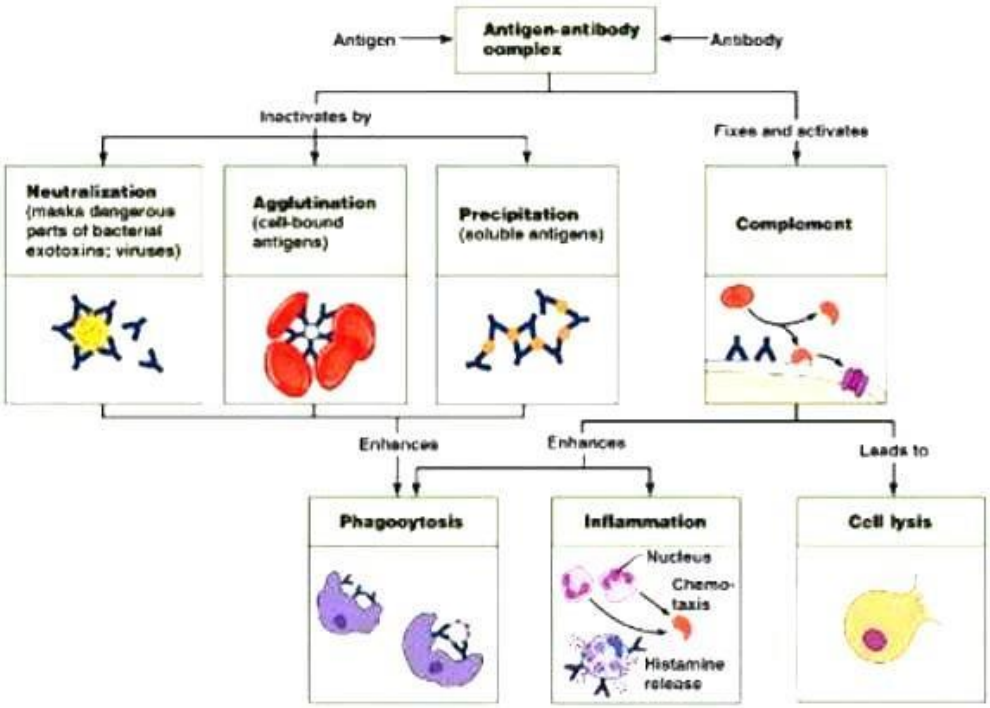


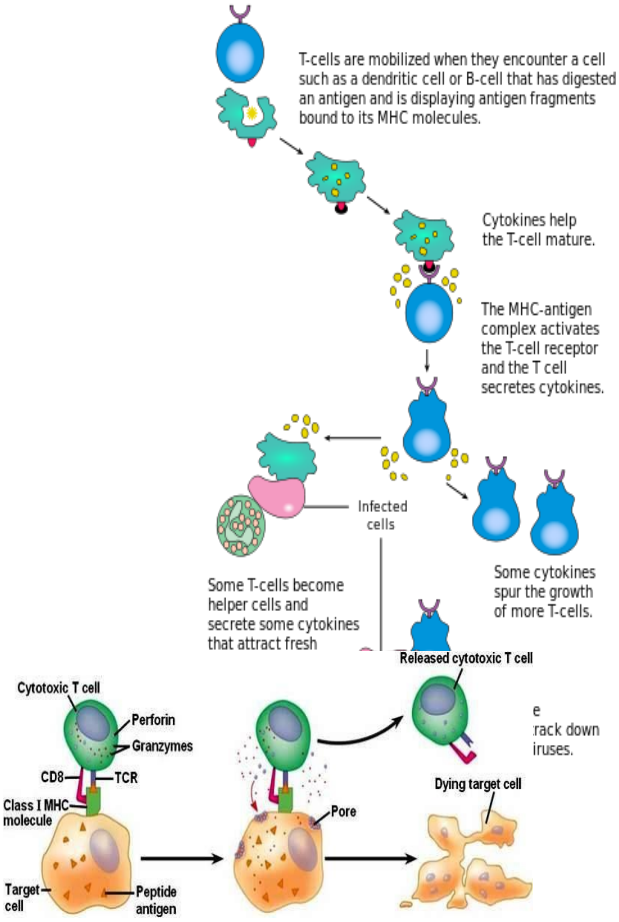
Antibody Targets and Functions

- **Complement fixation:** Foreign cells are tagged for destruction by phagocytes and complement fixation Immune complex formation exposes a complement binding site on the C region of the Ig and Complement fixation results in cell lysis.
- **Neutralization**: immune complex formation blocks specific sites on virus or toxin & prohibit binding to tissues (antibodies block active sites on viruses and bacterial toxins so they can no longer bind to receptor cites on tissue cells and cause injury)

- **Agglutination**: cells are cross-linked by immune complexes & clump together
- **Precipitation**: soluble molecules (such as toxins) are cross-linked, become insoluble, & precipitate out of the solution
- **Inflammation & phagocytosis** prompted by debris

Antigen-Antibody Complex – Functions





Memory B cells are stimulated to multiply but do not differentiate into plasma cells; they provide the immune system with long-lasting memory.

***Cell-mediated immune response* (within the cell) -** does not involve antibodies but rather involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen

T cells – are produced in bone marrow but mature in the thymus gland T cells contribute to immune defenses in two major ways: some direct and regulate immune responses; others directly attack infected or cancerous cells.

Helper T cells – assist other white blood cells in the immunologic process including maturation of B cells into plasma cells and memory B cells and activation of T cells and macophages

Cytotoxic T cells – sometimes called killer T cells destroy virally infected cells and tumor cells and play a role in transplant rejection

Memory T cells –antigen-specific T cells the persist long-term after an infection has been resolved that will provide memory of past infection and earlier defense for new infection

Regulatory T cells – formally called **suppresser T cells** maintain balance by shutting down T-cell mediated immunity toward the end of an immune reaction – they are a self check built into the immune system to prevent excessive reactions. They play a key role in prevent autoimmunity

Antigens are proteins or carbohydrate chain of a glycolprotein within in plasma membrane that the body recognizes as ***nonself***

The antigens on the cell membrane of the target or invader cell are recognized

MHC (a protein marker on body's cell) binds to the antigen of the foreign cell forming an MHC complex

The MHC complex alerts the T cells about an invasion, macrophage, virgin B cell or cell infected by a microbe that displays the antigen on its membrane

The MHC complex activates the T cell receptor and the T cell secretes cytokines

The cytokines spur the production of more T cells

Some T cells mature into **Cytotoxic T cells** which attack and destroy cells infested with viruses or cancerous cells

- **Cytotoxic T cells** or **Killer T cells (NKT)** share the properties of both T cells and natural killer (NK) cells. They are T cells with some of the cell-surface molecules of NK cells. The kills cancer cells, cells that are infected (particularly with viruses), or cells that are damaged in other ways -They have storage granules containing porforin and granzymes (proteins which perforates the cell membrane of the cell to be destroyed allowing water & salts to enter and rupture the cell). They and are implicated in disease progression of asthma and in protecting against some autoimmune diseases, graft rejection, and malignant tumors
- Other T cells mature into **Helper T cells** which regulate immunity by increasing the response of other immune cells
- **Helper T cells** secrete **cytokines** (messenger molecules) when exposed to antigens that causes more Helper T cells to be cloned, B cells to make antibodies and macrophages to destroy cells by phagocytosis
- AID's virus attacks to Helper T cells so it inactivates the immune system
- **Regulatory T cells** will shut down T-cell mediated immunity when things are under control
- **Memory T** cells persist sometimes for life and protect in case of re-infection

T-cell maturation, activation and differentiation

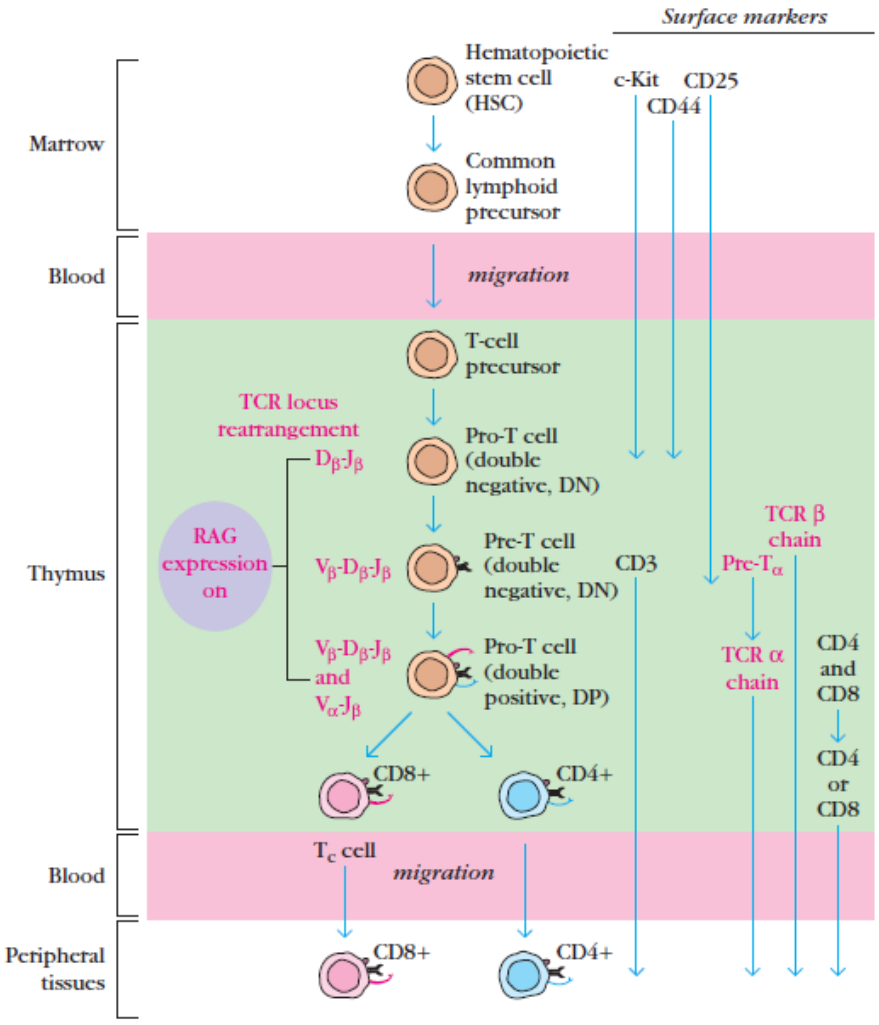
T-cell maturation:

- The migration of progenitor T-cells from the early sites of hematopoiesis to the thymus takes place at about day 11 of gestation in mice and in 8^{th} or 9^{th} week of gestation in humans.
- T-cell maturation involves the re-arrangement of the germ-line TCR genes and the expression of various membrane markers.
- In thymus, the developing T cells are termed as **thymocytes.**
- These thymocytes proliferates and differentiates along developmental pathways that produce functionally distinct sub-population of mature T-cells.
- T-cells development initiates with the arrival of small numbers of lymphoid precursors migrating from the blood into the thymus where they proliferate, differentiate and undergo selection process that result in the development of mature T cells.
- When T-cells precursor arrive at thymus, they don't express the signature surface markers of T cell as the T-cell receptors, the CD_3 complex or the co-receptors CD_4 and CD_8.
- In-fact these progenitor cells have not yet re-arranged their TCR genes and do not express the proteins, such as RAG-1 and RAG-2 that are required for re-arrangement.
- After arriving in the thymus T cell precursors enter the cortex and slowly proliferate.
- The differentiating T-cell pass through a series of stages that are marked by characteristic changes in their cell surface phenotype, during approximately three weeks of development in thymus.
- The thymocytes early in the development lack detectable CD_4 AND CD_8.
- As these cells are CD_4-, CD_8-, they are termed as double negative (DN) cells.
- In-fact DN-T cells can be sub-divided into 4 subsets (DN1-4) characterized by the presence or absence of cell surface molecules in addition to CD_4 and CD_8, such as:
    - **C-kit,** the receptor for stem cell growth factors
    - **CD_{44},** an adhesion molecule

- **CD_{25}**, it is the alpha chain of the IL-2 receptor

- The cells that enter the thymus DN1 cells are capable of giving rise to all the subsets of T cells and are phenotypically C-kit^{+}, CD_{44} high and CD_{25}-.
- Once the DN_1 cells encounter the thymic environment, they begin to proliferate and express CD_{25} becoming C-kit^{+}, CD_{44} high and CD_{25}+.
- These cells are called DN_2 cells.
- During the critical DN_2 stage of development, rearrangement of the genes for the TCR, ***Y, δ*** and ***β*** chain begins.
- However, the TCR ***α*** locus does not re-arrange, presumably because the region of DNA encoding TCR ***α*** gene is densely compacted and not accessible to recombinase machinery.
- As cells progress to DN_3, the expression of both C-kit and CD_{44} is turned off and TCR ***Y***, TCR ***δ***, and TCR ***β*** re-arrangement progresses.
- Cells destined to become ***Yδ*** T cell, diverge at the transition between DN_2 and DN_3 and become mature.
- ***Yδ*** T cell which vary few changes in their surface phenotype most DN_2 cells are destined to give rise to T cells.
- On assuming the DN_3 phenotype (C-kit^{-}, CD_{44}– and CD_{25}+), the cells halt proliferation and protein products of TCR ***β*** rearrangements are detected in the cytoplasm of these cells.
- The newly synthesized ***β***-chain combine with 33-kDa glycoprotein known as the pre-T cell receptor or pre-TCR.
- Formation of pre-TCR activates a signal transduction pathway that has the following consequences:
- It indicates that a cell has made a productive TCR ***β***-chain re-arrangement and signals its further proliferation and maturation.
- It suppresses further re-arrangement of TCR ***β***-chain gives resulting in the allelic exclusion.
- It renders the cell permissive for re-arrangement of the TCR ***α*** chain.
- It induces developmental progression to CD_{4+} and CD_{8+} double positive (DP) state.
- After ***β***-chain rearrangement is completed the DN_3 cells quickly progress to DN_4, the level of CD_{25} falls and both CD_4 and CD_8 co-receptors are expressed.

- Thus, double positive (DP) stage is a period of rapid proliferation.
- However, TCR ***α***-chain gene rearrangement still has not occurred at this stage of time.
- The rearrangement of ***α***-chain gene does not begin until the double positive (DP) thymocytes stops proliferating and RAG-2 protein level increases.
- T-cell development is a costly process for the host.
- An estimated 98% of all thymocytes do not mature i.e. they die by apoptosis within the thymus either because they fail to make a productive TCR gene re-arrangement or because they fail to survive thymic selection.
- Double positive thymocytes that express ***αβ*** TCR CD_3 complex and survive thymic selection develop into immature single positive CD_4+ thymocyte or single positive CD_8+ thymocytes.
- These single positive cells undergo additional negative selection and migrate from the cortex to medulla, where they pass from the thymus into the circulatory system.
- After the gene rearrangement, T-cell (Thymocytes) undergoes thymic selection.

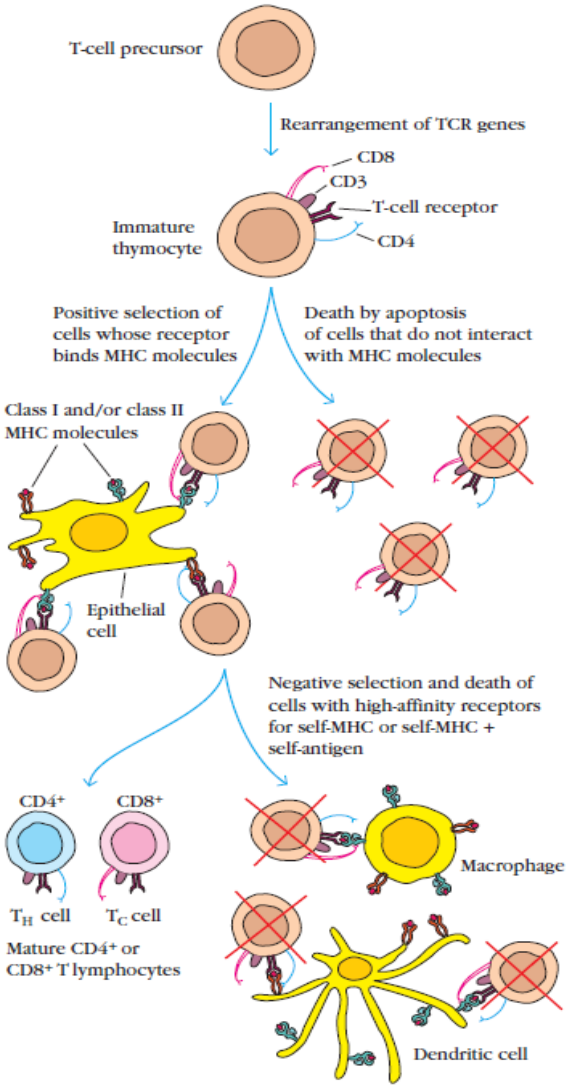


Thymic selection of T-cell:

- The most characteristic property of mature T-cells is that they identify only foreign antigen combined with self MHC molecules.
- For this purpose, the thymocyte undergo two selection process in thymus.
- **Positive selection:**
    - Positive selection occurs in the cortical region of the thymus.
    - It involves the interaction of immature thymocytes with cortical epithelial cells.
    - This interaction allows the immature thymocytes to receive a protective signal.
    - This signal prevents them from undergoing cell death.

- Cells whose receptors are not able to bind MHC molecules would not encounter with the thymic epithelial cells and as a result it would not receive the protective signal resulting their death by apoptosis.
- This results in MHC restriction.

- **Negative selection:**
  - The population of MHC restricted thymocyte that survive positive selection includes cells with receptors having a range of affinities from low to high for self-antigen presented by self-MHC molecule.
  - Thymocytes with high affinity receptors are weeded out during negative selection via an interaction with thymic stomal cells.
  - In case of negative selection, dendritic cells and macrophages having class I and class II MHC molecules interact with thymocyte bearing high affinity receptors for self MHC molecule alone.
  - cells that undergo negative selection are observed to undergo death by apoptosis.
  - Tolerance to self-antigens encountered in the thymus achieved by eliminating T-cells that are reactive to these antigens.



Thymic selection of T cells

T-cell activation:

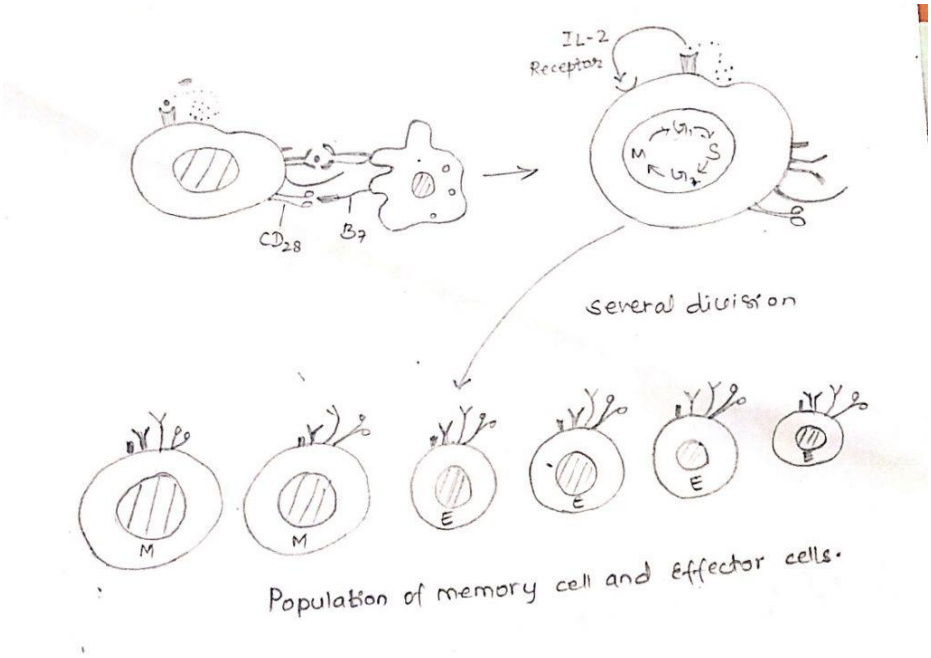
- The central event in the generation of both humoral and cell-mediated immune responses in the activation and clonal expansion of T-cells.
- T-cells activation is initiated by interaction of the TCR-CD_3 complex with a processed antigenic peptide bound to either class-I (CD_8+ cell) or class II (CD_4+ cell) MHC molecules on the surface of an antigen presenting cells.
- The interaction and the resulting activating signals also involve various accessory membrane molecules on the T-cell and the antigen presenting cell.
- Interaction of T cell with antigen initiates cascade of biochemical event that induces the resting T-cell to enter the cell cycle, proliferating and differentiating into memory cells or effector cells.

- The key element in the initiation of T cell activation is the recognition by the TCR of MHC peptide complexes on antigen presenting cells.
- This event catalyses a series of intracellular events beginning at the inner surface of the plasma membrane and culminating in the nucleus, resulting in the transcription of genes that drive the cell cycle and/or differentiation of T-cell.

T-cell differentiation:

- CD_4+ and CD_8+ T-cells leave the thymus and enters the circulation as resting cells in the G_0 stage of cell cycle.
- There are about twice as many CD_4+ T-cells as CD_8+ T-cells in the periphery.
- T cells that have not yet encountered antigen (naive T-cells) are characterized by condensed chromatin, very little cytoplasm, and little transcriptional activity.
- Naive T cells continually reticulate between the blood and the lymph system.
- During recirculation, naïve T cells reside in secondary lymphoid organs/tissues such as lymph nodes.
- If a naive cell does not encounter antigen in a lymph node, it exits through the efferent lymphatics ultimately draining into the thoracic duct and rejoining the blood.
- It is estimated that each naive T cell recirculates from blood to the lymph nodes and back again every 12-24 hrs.
- This large-scale recirculation increases the chances that naive T cell will encounter appropriate antigen because only about 1 in 10^5 naive T -cell is specific for any given antigen.
- If naive T cell recognize an antigen-MHC complex on an appropriate antigen cell or target cell, it will be activated, initiating a primary response.
- About 48hrs after activation the naïve T cells enlarges into a blast cell and begins undergoing repeated rounds of cell-division.
- Activation depends on a signal induced by the enlargement of the TCR complex and a stimulatory signal induced by the CD_{28}-B_7 interaction.
- These signals trigger entry of T cell into the G_1 phase of the cell cycle and at the same time, induce transcription of the gene for IL-2 and α chain of the high affinity IL-2 receptor (CD_{25}).

- Moreover, the co-stimulatory signal increases the half-life of the IL-2 mRNA.
- The increase in IL-2 transcription together with the stabilization of the IL-2 mRNA, increases IL-2 production by 100 folds in the activated T-cell.
- Secretion of IL-2 and its subsequent binding to the high affinity IL-2 receptor induces the activated naïve T cell to proliferate and differentiate.
- T-cells activated in this way divide 2-3 times per day for 4-5 days generating a clone of progeny cells which differentiates into memory or effectors T-cell populations.



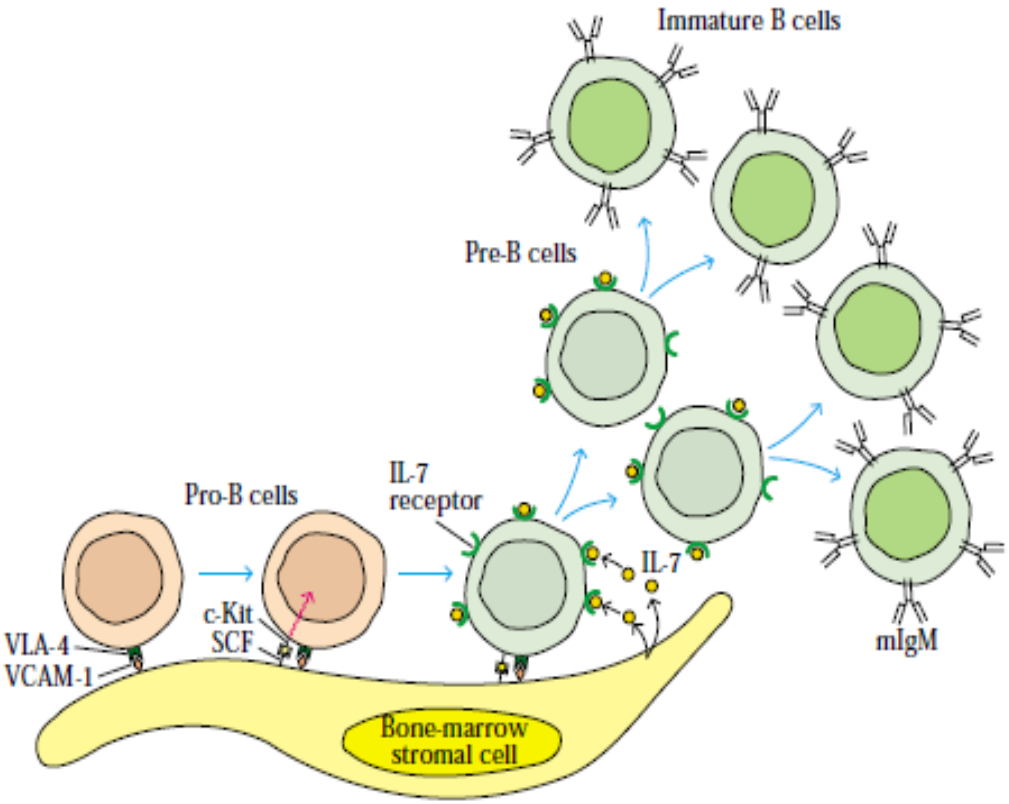
T cells differentiation

B-cell development: Maturation, activation and differentiation

B-cell development:

- The development of plasma cell and memory B cells can be divided into three broad stages:
- Generation of mature, immunocompetent B-cells (maturation)
- Activation of mature B-cells and the differentiation of the activated B-cells, into plasma cells and memory B cells.
- These three stages can be divided into two phases:

1. **Antigen independent phase:**
    - This takes place in bone marrow.
    - It involves the maturation of lymphoid progenitors to matured naive B cells.
2. **Antigen dependent phase:**
    - This takes place in lymph node.
    - It involves activation of mature B-cells then they encounter antigen and their differentiation into plasma cells and memory B-cells.



B cell development

B-cell maturation:

- The generation of B-cell first occurs in embryo and continues throughout life.
- Before birth, the yolk sac, foetal liver and foetal bone marrow are the major sites of B cell maturation.
- After birth, the generation of mature B-cells occur in the bone marrow from hematopoietic stem cells (HSC).
- The HSC first divide to form lymphoid progenitor cells which then differentiate into the progenitor B-cells (pro B) which express a transmembrane tyrosine phosphatase called CD45R and signal transducing molecule Igα/ Igβ which are found associated with the membrane bound antibody in later stages of development.
- Pro-B cell also express CD19 (part of co-receptor), CD43 (leukosialin), CD24 (heat stable), and C-kit are present on the surface of Pro-B-cell.
- The pro-B-cells proliferate within bone marrow filling extravascular spaces between large sinusoids in the shaft of a bone proliferation of pro-B-cells to

precursor-B-cells (pre-B-cell) require micro-environment provided by the bone marrow stromal cells.

- The stromal cell plays two important roles, they interact directly with Pro-B cell and Pre-B cell and they secrete various cytokines, notably IL-7 that support developmental process.
- Pro-B-cells need direct contact with stromal cells in the bone marrow during the earliest developmental stage.
- This interaction is mediated by several cell adhesion molecules including VLA-4 on Pro-B cell and its ligand, VCAM-1, on the stromal cell.
- After initial contact is made, a receptor on Pro-B cell called C-kit interacts with a stromal cell surface molecule known as stem cell factor (SCF).
- This interaction activates C-kit, a tyrosine kinase and Pro-B cell begins expressing receptor for IL-7.
- The Pre-B-cell express many of same marker that were present on Pro-B-cell, however they cease to express C-kit and CD43 and begin to express CD25.
- The IL-7 secreted by stromal cells drives the maturation process eventually inducing down the regulation of adhesion molecule on Pre-B cell.
- So, the proliferating cell can detach from stromal cells.
- At this stage, Pre-B-cell no longer requires direct contact with stromal cell but continues to requires IL-7 for growth and maturation.

Ig-gene re-arrangement producing immature B-cells:

- B-cell maturation depends on rearrangement of immunoglobulin DNA in the lymphoid stem cells.
- The first Ig-gene re-arrangement to occur in Pro-B-cell stage is a heavy chain DH-JH gene re-arrangement, this is VH-DH-JH rearrangement.
- If the first heavy chain rearrangement is not productive, then VH-DH-JH rearrangement continues on the other chromosome.
- Upon completion of heavy chain arrangement, the cell is classified as Pre-B-cell.
- Continued development of a Pre-B-cell into an immature B-cell requires a productive light-chain gene re-arrangement.

- Only one light chain isotype is expressed on the membrane of a B-cell because of allelic exclusion.
- Upon completion of productive light chain re-arrangement, it commits the immature B-cell to a particular antigenic specificity.
- This specificity is determined by the cells heavy chain VDJ sequence and light chain VJ sequence.
- Immature B cell expresses mIgM on its cell surface.
- The bone marrow phase of B-cell development culminates in the production of IgM bearing immature B-cell.
- At this stage of development, B-cell is still not fully functional.
- Thus, antigen induces death or unresponsiveness rather than division and differentiation.
- The co-expression of IgD and IgM on the membrane signals the full maturation.
- This progression involves a change in RNA processing of the heavy chain primary transcript to permit the production of two mRNAs, one encoding the membrane form of the μ chain and other encoding the membrane of the δ chain.

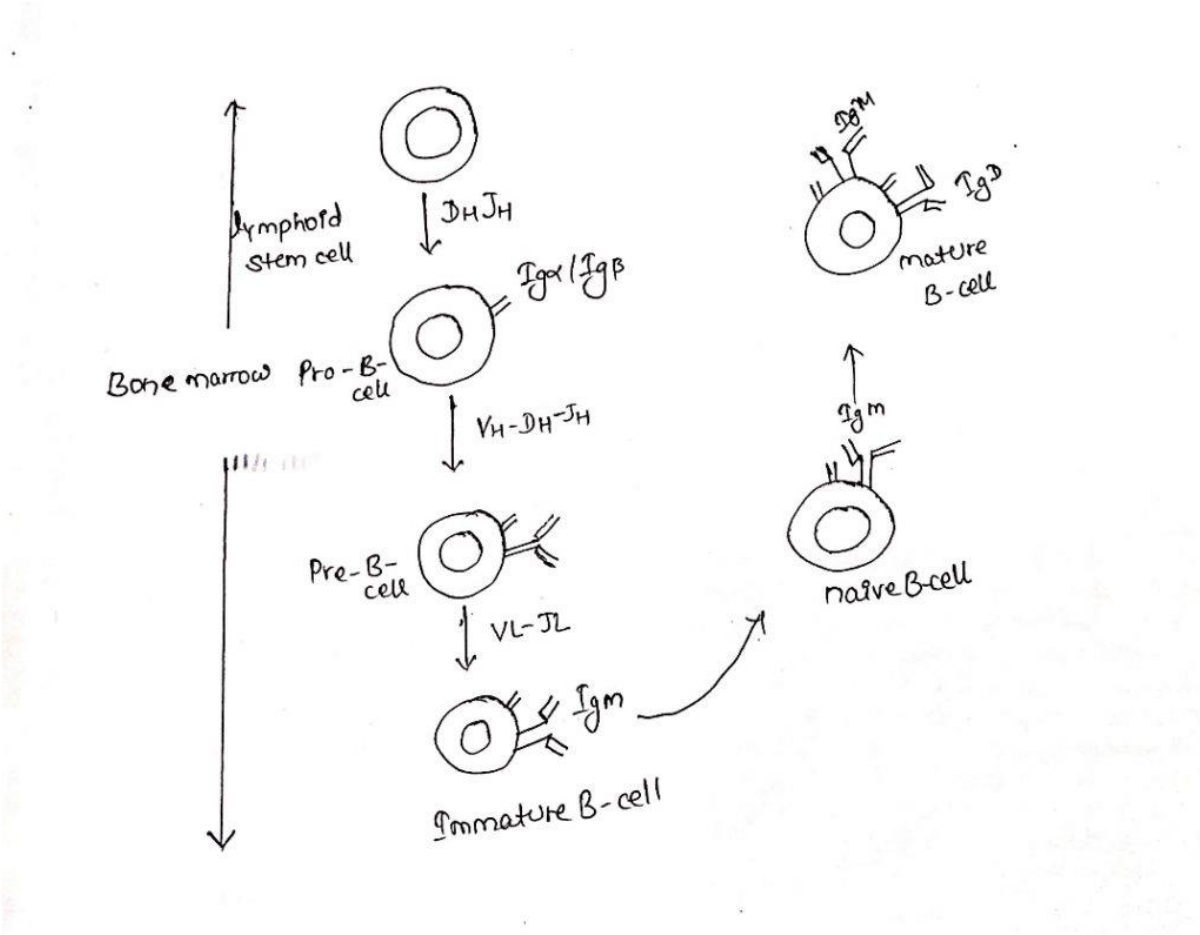


Fig. B cell maturation

B-cell proliferation and activation:

- After export of B-cell from the bone-marrow, activation, proliferation and differentiation occur in the periphery and require antigen.
- Depending on the nature of the antigen, B cell activation proceeds by two different routes, one dependent of TH cell, the other not.
- The B cell response to thymus dependent (TD) antigen requires direct contact with TH cell, not simply exposure to TH derived cytokines.
- Antigens that can activate B cells in absence of this kind of direct participation by TH cells are known as thymus independent (TH) antigen.
- The TI antigens are divided into two types 1 and 2 and they activate B-cells by different mechanisms.
- Most TI1 antigens are polyclonal B cell activator i.e. they are able to activate B-cell regardless of their antigenic specificity.

- At high concentration TI-1 antigens will stimulate proliferation and antibody secretion by as many as one third of B-cells.
- It includes bacterial cell wall components including lipopolysaccharide.
- B cells are activated by TI-2 antigens by extensively crosslinking the mIg receptor.
- However, TI-2 antigens contrasts to TI-1 antigens in three important respects.
- First, they are not B-cell mitogens and do not act as a polyclonal activators.
- Second, TI-1 antigens activate both mature B-cells and immature B cells. Whereas TI-2 antigen activates mature B cells and inactivates immature B-cells.
- Third, although B cell response to TI-2 antigen does not require direct involvement of TH cells, cytokines derived from TH cells are required for efficient B-cell proliferation and for class switching to isotypes other than IgM.
- It includes highly repetitious molecules like bacterial flagellin.
- Activation of B-cell by soluble protein antigen requires the involvement of TH cells.
- Binding of antigen to B-cell mIg does not itself induce on effective competence without additional interaction with membrane molecule on the TH cell.
- In addition to it, a cytokine mediated progression is required for B-cell proliferation.

i. Formation of T-B conjugate:

- After binding of antigen by mIg on B cell, the antigen is internalized by receptors mediated endocytosis and processed within the endocyte pathway into peptide.
- Antigen binding also initiates signaling through the BCR that induces the B-cells to upregulate a no. of cell membrane molecules, including class II MHC molecules and co-stimulatory ligand B7.
- Increased expression of both of these membrane proteins enhance the ability of B-cell to function as an antigen presenting cell (APC) in TH cell activation.
- Once the TH cell recognizes a processed antigenic peptide displayed by a class II MHC molecule on the membrane of B-cells, the two cells interact to form a T-B conjugate.

- This structural adjustment facilitates the release of cytokines towards the antigen specific B-cells.

ii. CD40-CD40L interaction:

- Formation of a T-B conjugate not only leads to directional release of TH cell cytokines but also to the upregulation of CD40L, a TH cell membrane protein that then interacts with CD40 on the B-cell to provide essential signal for T-cell dependent B-cell activation.
- Interaction of CD40L with CD40 on the B-cell delivers a signal (signal-2) to the B-cell that in cooperation with the signal generated by **mIg** cross-linkage (signal1), drives the B cell into G1.
- The signal from CD40 are transducted by a no. of intracellular signaling pathway, ultimately resulting in changes in gene expression.

iii. Signals provided by TH cell cytokines:

- The antigenic specific interaction between a TH and a B cell induces a redistribution of TH cell membrane proteins and cytoskeletal elements that results in the polarized release of cytokines towards the interacting B-cell.
- Once the B cell becomes activated, it begins to express membrane receptors for various cytokines such as IL-2, IL-4, IL-5 and others.
- These receptors then bind the cytokines produced by these cytokine-receptor interaction support B-cell proliferation and can induce differentiation, proliferation and can induce differentiation into plasma cells and memory cells, class switching and affinity maturation.
    - Antigen crosslinking mIg, generating signal 1 which results in increased expression of class II MHC and co-stimulatory B7. Antigen-antibody complexes are internalized by receptor mediated endocytosis. Then it is degraded to peptides, some of which are bound by class II MHC and presented on the membrane as peptide-MHC complexes.
    - TH cell recognizes antigen-MHC-II on B-cell membrane. This plus co-stimulatory signal activates TH cell.

- i. TH cell begins to express CD40L
  ii. Interactions of CD40 and CD40L provides signal 2
- iii. B7-CD28 interactions provide co-stimulation to the TH cell.
- i) B-cell begins to express receptors for various cytokines.
  ii) Binding to cytokines released from TH cell in a directed manner relays signal that the progression of the B-cell to DNA synthesis and to differentiation.

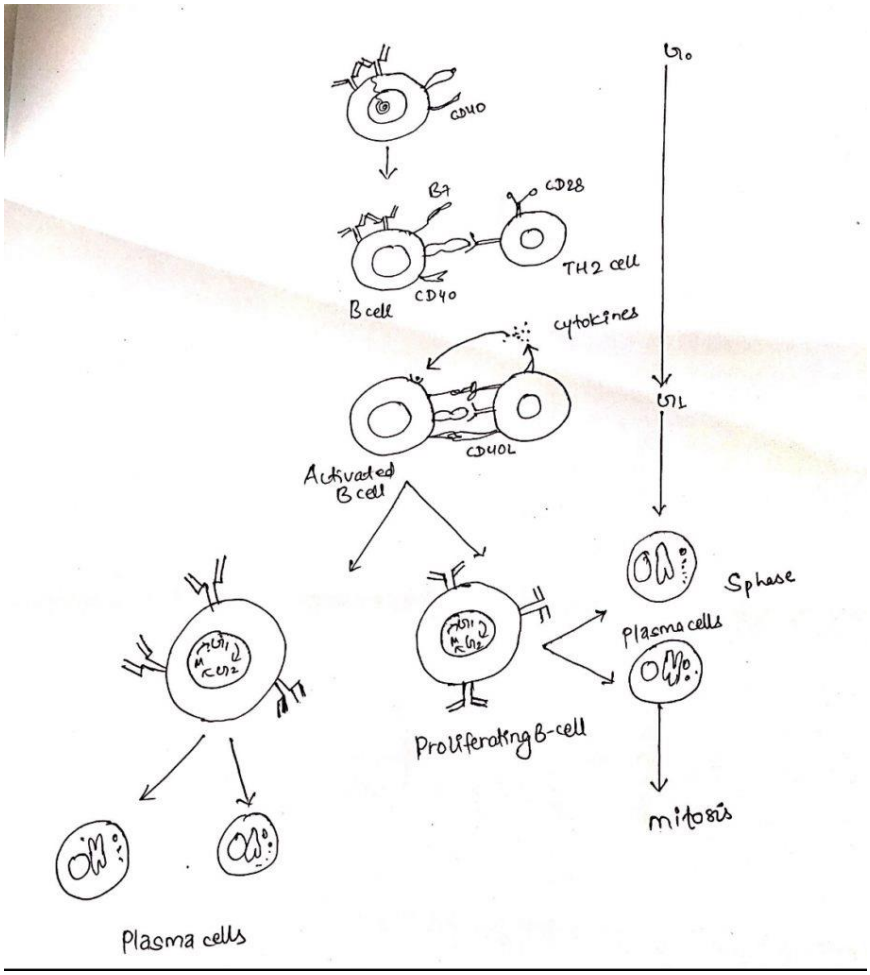


Fig. B cell differentiation

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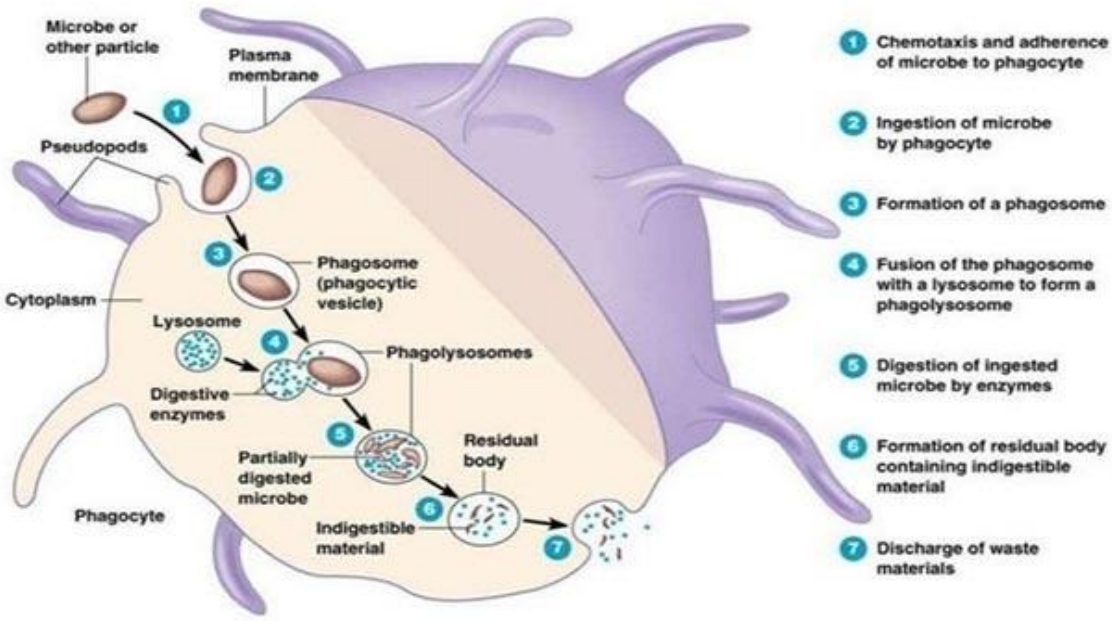
DEPARTMENT OF BIOTECHNOLOGY

UNIT – III – Cell Mediated Immunity – SBTA1402

Phagocytosis- definition, mechanism, steps with example

- Phagocytosis is a process by which cells ingest large particles (> 0.5 micrometers) into membrane-bound vesicles called phagosomes, which are then targeted to the lysosomes for enzymatic degradation.
- Phagocytosis is greatly enhanced by the opsonization of the bacteria.
- Attachment of a specific IgG or complement fragment, C3, can greatly enhance the efficiency of phagocytosis, although phagocytosis can occur without opsonization.
- Complement receptors, CR1 and CR3, are the primary receptors for opsonization by the complement.
- The Polymorphonuclear Leukocytes (PMNs) also express receptors for IgG fragment Fc (FcγRs) that facilitates phagocytosis.
- The two most prominently expressed Fcγ receptors on circulating PMNs are known as FcγRII (CD32) and FcγIII (CD16), and binding to these receptors triggers the oxidative burst in PMNs.
- The binding of antibody and complement receptors at the PMNs surface activates the phagocytic process.



Mechanism of Phagocytosis

Destruction of the Microbes

The following are important factors that help destroy microorganisms within a phagolysosome:

Oxygen Radicals: A complex of proteins called phagocyte oxidase in the membrane of a phagolysosome generates oxygen radicals in the phagosome. A single electron is taken from NADPH and added to oxygen, partially reducing it. The resulting highly reactive molecules react with proteins, lipids and other biological molecules.

Nitric Oxide: Nitric oxide synthase synthesizes nitric oxide, a reactive substance that reacts with superoxide to create further molecules that damage various biological molecules.

Anti-Microbial Proteins: Lysosomes contain several proteases, including a broad spectrum enzyme, elastase, which is important or even essential for killing various bacteria. Another anti-microbial protein is lysozyme, which attacks the cell walls of certain (gram-positive) bacteria.

Anti-Microbial Peptides: Defensins and certain other peptides attack bacterial cell membranes. Similar molecules are found throughout much of the animal kingdom.

Binding Proteins: Lactoferrin binds iron ions, which are necessary for the growth of bacteria. Another protein binds vitamin B12.

Hydrogen Ion Transport: Transporters for hydrogen ions (a second role of the oxidase) acidify the phagolysosome, which kills various microorganisms and is important for the action of the proteases described above.

In addition to destroying the microorganism, phagocytes also release molecules that diffuse to other cells and help coordinate the overall response to an infection.

Regulatory molecules that regulate an immune response are called **cytokines**. Most are small proteins and are mainly released by white blood cells and their relatives, such as macrophages.

Several types of cells in the immune system engulf microorganisms via phagocytosis.

Neutrophils: Neutrophils are abundant in the blood, quickly enter tissues, and phagocytize pathogens in acute inflammation.

Macrophages: Macrophages are closely related to monocytes in the blood. These longer-lived cells predominate in chronic inflammation. They also release some important inflammatory paracrine.

Dendritic Cells: Phagocytosis in these cells is important for the elaboration of a specific immune response rather than for directly destroying the pathogens.

B Lymphocytes: A small amount of phagocytosis in these cells is often necessary in order for them to develop into cells that release antibodies.

- The PMNs have two broad types of killing mechanisms.
- One is oxygen-dependent and the other is oxygen-independent.
- Phagocytosis of microbes stimulates the production of superoxide radicals and other reactive oxygen species.

- These are potent microbicidal agents and include, among others, hydrogen peroxide and chloramines.
- The enzyme NADPH oxidase is found in the cell membrane of the PMNs and generates the superoxide (called the respiratory burst).
- The superoxide is unstable and quickly dismutates to hydrogen peroxide and other substances that are microbicidal.
- These reactions take place inside the phagolysosome (also called phagosome).

Steps in the phagocytosis of a bacterium

1. Bacterium becomes attached to membrane evaginations called pseudopodia.
2. The bacterium is ingested, forming a phagosome.
3. Phagosome fuses with a lysosome.
4. The bacterium is killed and then digested by lysosomal enzymes.
5. Digestion products are released from a cell.

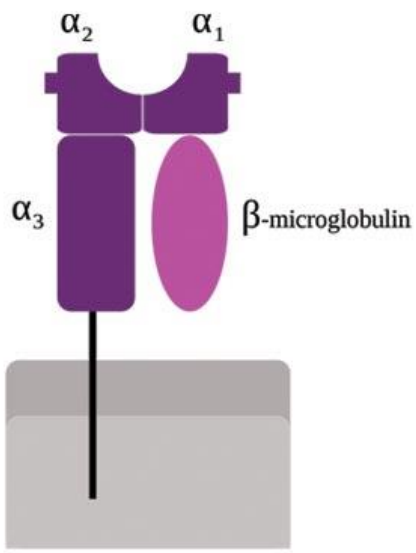
Example: Macrophage phagocytizing a virus.

1. The virus and the cell need to come into contact with each other.
2. The virus binds to the cell surface receptors on the macrophage.
3. The macrophage starts to invaginate around the virus, engulfing it into a pocket.
4. The invaginated virus becomes completely enclosed in a bubble-like structure, called a “phagosome”, within the cytoplasm.
5. The phagosome fuses with a lysosome, becoming a “phagolysosome”.
6. Phagolysosome lowers the pH to break down its contents.
7. Once the contents have been neutralized, the phagolysosome forms a residual body that contains the waste products from the phagolysosome.
8. The residual body is eventually discharged from the cell.

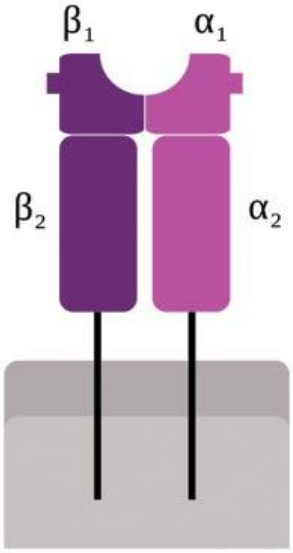
Major Histocompatibility Complex (MHC)

Definition

The major histocompatibility complex can be defined as a tightly linked cluster of genes whose products play an important role in intercellular recognition and in discrimination between self and non-self. The term ‘histo’ stands for tissue and ‘compatibility’ refers to ‘getting along or agreeable’. On the other hand, the term ‘complex’ refers to the ‘genes that are localized to a large genetic region containing multiple loci’. These genes code for antigens which involve in the determination of the compatibility of the transplanted tissue. The compatible tissues will be accepted by the immune system while the histo-incompatible ones are rejected. The rejection of foreign tissue leads to an immune response to cell surface molecules. The concept was first identified by Peter Gorer and George Snell. The main function of MHC molecules is to bring antigen to the cell surface for recognition by T cells. In humans, the genes coding for MHC molecules are found in the short arm of chromosome 6.



Class I MHC Molecule



Class II MHC Molecule

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) TYPES

In humans, the MHC molecules are divided into three types, Class I, Class II and Class III. Class I MHC molecules are coded from three different locations called A, B and C and these molecules are expressed in all nucleated cells. Class II MHC genes are located in the D region and there are several loci such as DR, DQ and DP and these molecules are expressed only in antigen-presenting cells. Class III MHC genes are coded in the region between Class I and Class

II genes. Class III MHC genes codes for cytokines and complement proteins which play an important role during the immune response.

Class I MHC Molecule

- The structure of Class I MHC molecule consists of two polypeptide chains α and β. These two chains are connected together by non-covalent bonds. The α chain is characterized as an internal membrane glycoprotein with a molecular weight of 45000 Da (in humans). B chain, on the other hand, is an extracellular microglobulin with a molecular mass of 12kDa.
- The α chain is made up of approximately 350 amino acids and also divided into three globular domains α_1, α_2 and α_3. Each of these domains contains roughly 90 amino acids. The N terminal of α chain is the place of α_1 domain, while α_2 and α_3 are present after α_1 The α_2 domain is characterized by the formation of a loop of 63 amino acids; the loop is formed due to intrachain disulfide bond. α_3 also contains a disulfide bond enclosing 86 amino acids. The α_1 and α_2 domains interact to form peptide-binding units of class I MHC molecule.
- Moreover, α chain also consists of a stretch of 26 hydrophobic amino acids which holds the α chain on the plasma membrane. This transmembrane segment is present as a form of α helix at the hydrophobic region of the plasma membrane. An intracellular domain or the carboxyl-terminal of α chain is located inside the cell and it contains around 30-40 amino acids.
- In humans the β chain is non-polymorphic and it is dimorphic in mice. α_3 and β chain are structurally similar to the immunoglobulin C domain and also characterized as a disulfide loop. A peptide binding platform is formed by β plated sheets of α_1 and α_2
- T_{cyt} Cell (cytotoxic T cell) has specificity towards cells containing peptides associated with Class I MHC due to the presence of CD8 antigen on the surface of Tcyt Cell. CD8 antigen has an affinity towards the α_3 domain of Class I MHC molecules.

Class II MHC Molecule

- Class II MHC molecules are heterodimers and characterized by two non covalently connected polypeptide chains. The chains are termed as a heavy chain (α, 30kDa) and light chain (β, 26kDa).
- Similar to class I MHC molecules, class II MHC molecules are also characterized by an extracellular amino terminal domain, a transmembrane domain and an intracellular carboxy terminal tail.
- The class II MHC molecules are expressed on the surface of the antigen-presenting cells such as B cells, dendritic cells, and macrophages.

• The α chain is divided into two domains α_1 and α_2, while the β chain is also divided into two groups β_1 and β_2. The β_2 domain is responsible for the binding of T cell co-receptor CD4. The α_1 and β_1 domain, on the other hand, involved in the formation of the antigen-binding sites. Peptides containing 13-20 amino acids can bind at the antigen-binding site of class II MHC.

• The presence of disulfide bonds in α_2, β_1, and β_2 domains are also an important structural feature of the class II MHC molecules.

Class III MHC Molecule

• There are several serum proteases which involve in compliment system come under the group of class III MHC molecules.

• Class III MHC molecules do not have any involvement in antigen presentation.

• The complement components such as asC2, C4A, and C4B, and factor B are the most important compounds involve as class III MHC molecules. Apart from these tumor necrosis factors α and β and some heat shock proteins also come under this category.

ANTIGEN PRESENTATION AND PROCESSING

The T cells can recognize the foreign antigen when the antigen is attached to the MHC molecules as an MHC peptide complex. The formation of the MHC-peptide complex requires the degradation of protein antigen by several steps. The degradation process is known as antigen processing. These degraded proteins are then attached to the MHC molecules inside the cell and then the MHC molecules transported to the membrane to present the antigen with the T cell.

Antigen Presentation Pathway: Class I MHC molecules (Cytosolic pathway)

• Class I MHC molecules involve in presenting intracellular or endogenous pathogens or antigens. Intracellular pathogens refer to those organisms which live and replicates inside the host cell. An example of this type of pathogen is a virus.

• Under normal condition the MHC class I molecules forms a complex with the self-peptides or self-antigens. While, in case of any viral infection, the MHC class I molecules present the peptide derived from the virus which is then further recognized by T cells.

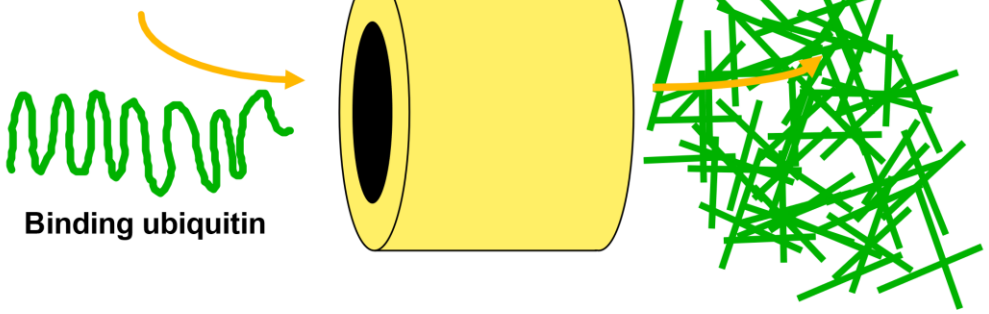
• Cell components such as a nucleus, endoplasmic reticulum and Golgi apparatus play an important role in antigen processing and presentation.

• When a virus infected a normal cell, the viral DNA moves inside the cell and produce viral proteins with the help of the host cell mechanisms. The viral proteins are synthesized in the cytosol.

- The cytoplasm also contains a cylindrical protein complex called the proteasome. The main function of the proteosome is to degrade the unwanted or damaged protein into smaller peptides. At the time of viral infection, the viral proteins interacted with the proteosomes present in the cytoplasm. The processing took place in the cytosol and as a result, the proteins are degraded into smaller peptides (8-15 amino acid long).
- In the next step, these fragmented peptides are transported into the endoplasmic reticulum. The transport took place due to a peptide delivery system called the transporter associated with antigen processing (TAP). TAP is made up of two domains or subunits called TAP 1 and TAP 2.
- Inside the endoplasmic reticulum the α and β chains of MHC class I molecules are synthesized and by the help of a group of chaperone proteins, the MHC class I molecule is formed and moves towards the TAP. As a result, the peptides bind at the peptide-binding site of the class I MHC molecule inside the endoplasmic reticulum and forms the MHC class I-peptide complex.
- In the next step, the MHC class I- peptide complex moves to the surface of the Golgi apparatus and by the help of secretory vesicle, it moves towards the surface of the plasma membrane.
- Once the MHC class I-peptide complex reaches the cell surface, the T cell receptors recognize the antigen peptide complex. Moreover, the co-receptor CD8 of the T cell attaches with the α_3 domain of the MHC class I molecule. Hence, the antigen is presented to the T cell.

Degradation in the proteasome

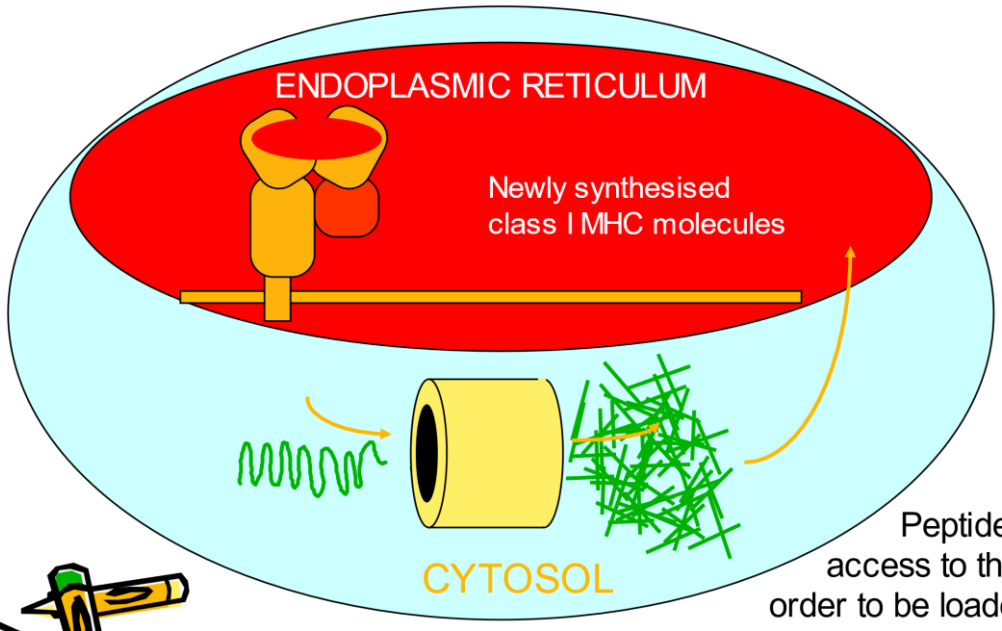
Cytoplasmic cellular proteins, including non -self proteins are degraded continuously by a multicatalytic protease



The components of the proteasome include LMP2, LMP7, MECL -1（LMP10）

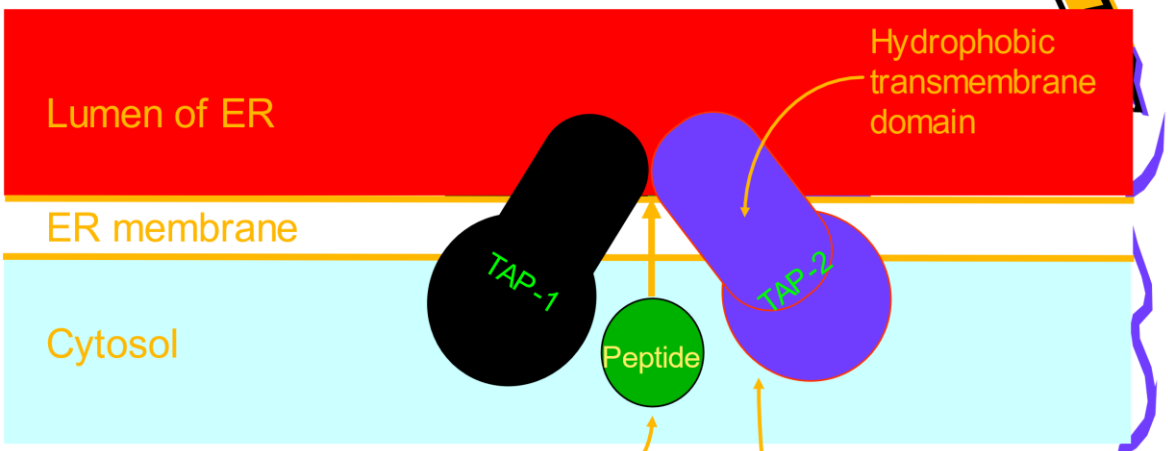
*MECL-1：Multicatalytic endopeptidase complex subunit

Peptide antigens produced in the cytoplasm are physically separated from newly formed class I MHC



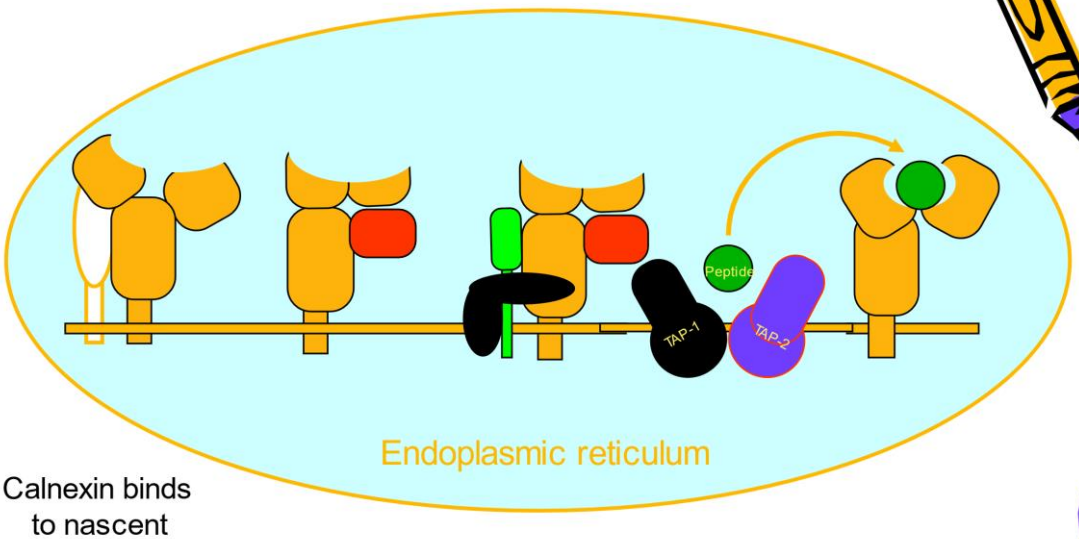
Peptides need access to the ER in order to be loaded onto class I MHC molecules

Transporters associated with antigen processing (TAP1 & 2)



Transporter has preference for >8 amino acid peptides with hydrophobic C termini.

Maturation and loading of class I MHC



Calnexin binds to nascent class Iα chain until β2-M binds

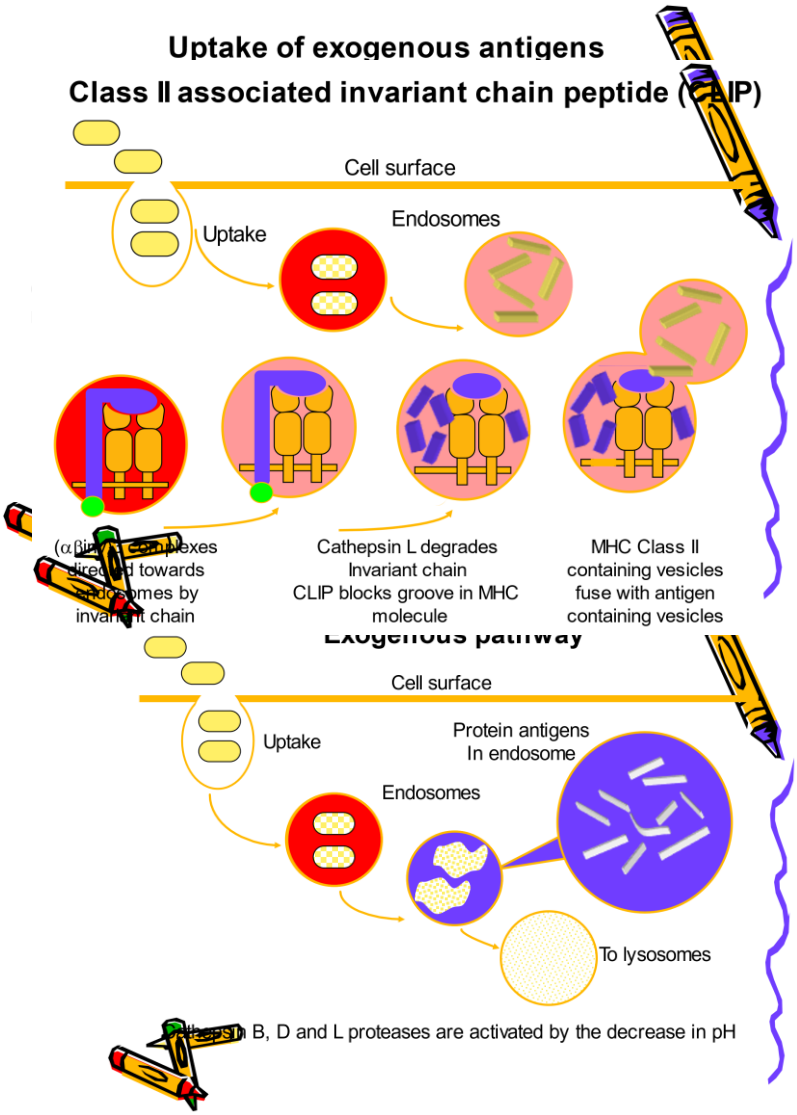
B2-M binds and stabilises floppy MHC

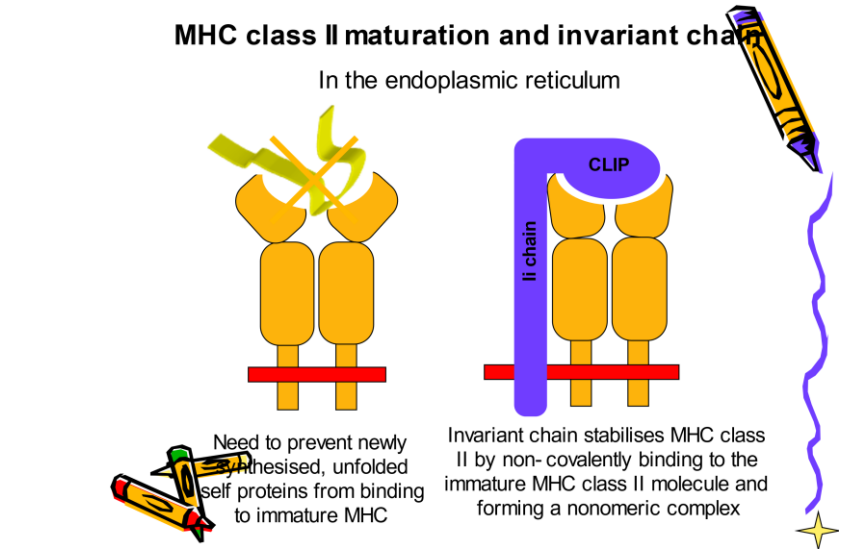
Tapasin, calreticulin, TAP 1 & 2 form a complex with the floppy MHC

Cytoplasmic peptides are loaded onto the MHC molecule and the structure becomes compact

Antigen Presentation Pathway: Class II MHC molecules (Endocytic Pathway)

- MHC class II molecules are responsible for presenting exogenous or extracellular pathogen or antigen. The extracellular pathogen refers to the organisms which can grow and reproduce outside of the host cell. Bacteria, exotoxins, parasites are examples of extracellular antigens. These antigens are taken up by the cell by endocytosis or phagocytosis.
- Only the antigen-presenting cells involved in antigen processing and presentation by MHC class II molecules. These cells include B cells, macrophages, and dendritic cells. The pathway took place only after the engulfment of the antigen by the antigen-presenting cells.
- Inside the cell, the antigen carries a covering called an endosome. The endosome is fused with the lysosome present in the cytoplasm and forms endolysosomes. As a result, the foreign protein is degraded by the proteolytic enzyme present inside the lysosome and small peptides are formed.
- The class II MHC molecules are synthesized and formed in the endoplasmic reticulum. The α and β chain of the molecule is also associated with the invariant chain. This association helps to restrict the binding of self-antigen with the class II MHC molecule. The invariant chain-MHC complex is then transported from the endoplasmic reticulum to the Golgi apparatus and from the Golgi apparatus to another vesicle. Inside the vesicle, the invariant chain is digested and only a small fragment (Class II-associated invariant chain polypeptide: CLIP) is attached with the molecule.
- In the next step, the vesicle containing the MHC class II molecule is then fused with the vesicle containing fragmented peptides. The fragmented peptide is then bound with the MHC class II molecule by displacing the CLIP. This newly formed MHC class II-peptide complex is then transported to the surface of the cell.
- Once at the cell surface, the antigen is presented to the T cells. The T cell recognizes the peptide bound with the MHC class II molecule by the help of the T cell receptor and the CD4 co-receptor binds with the β_2 domain of the class II MHC molecule.


Uptake of exogenous antigens
Class II associated invariant chain peptide (CLIP)
Cell surface
Uptake
Endosomes
Cathepsin L degrades
Invariant chain
CLIP blocks groove in MHC
molecule
MHC Class II
containing vesicles
fuse with antigen
containing vesicles
Cell surface
Uptake
Protein antigens
In endosome
Endosomes
To lysosomes


In the endoplasmic reticulum
CLIP
Ii chain
Invariant chain stabilises MHC class
II by non- covalently binding to the
immature MHC class II molecule and
forming a nonomeric complex

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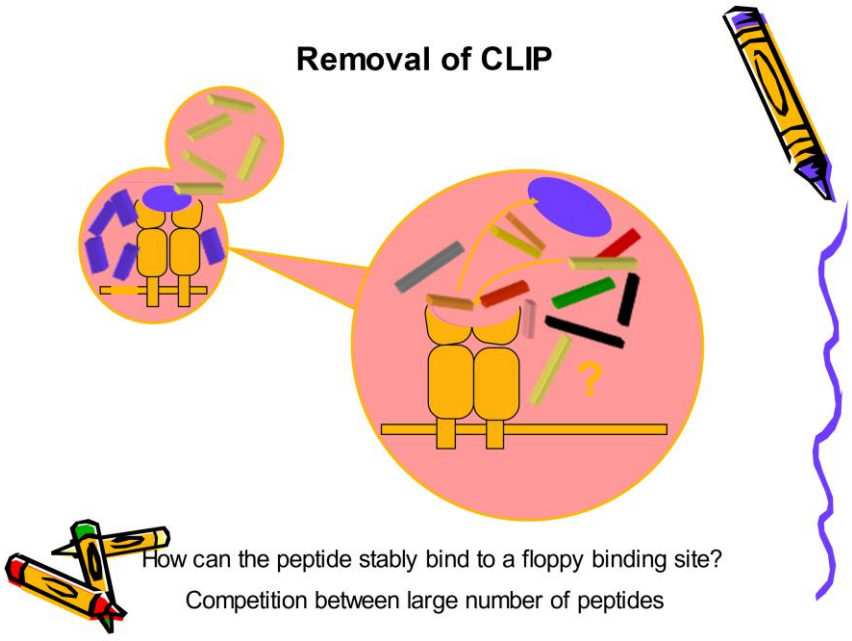
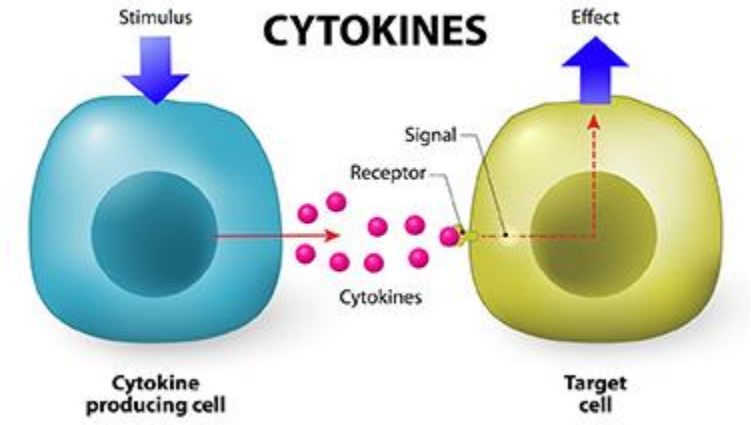

Removal of CLIP
?
How can the peptide stably bind to a floppy binding site?
Competition between large number of peptides

CYTOKINES



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Cytokines: properties and receptors

- Cytokines are low molecular weight (30KDa) regulatory protein or glycoprotein secreted by White blood cells and various other cells in body in response to number of stimuli. These regulatory proteins help in regulating the development of immune effector cells and some cytokines also have their own functions.
- Many cytokines are refered as interlukins; the name indicates that they are secreted by leucocytes and acts upon other leucocytes. Interlukins are designed from IL-1 to IL-29 and it is supposed that additional cytokines will be discovered and the group Interlukins will expand further.
- Some cytokines are known by their common name. for examples; tumor necrosis factor, Interferon etc.

Properties of cytokines:

1. Target Specific and induce signal transduction:

- cytokines binds to specific receptor on the cell membrane of target cell which triggers signal transduction pathway that ultimately alter gene expression in target cell.

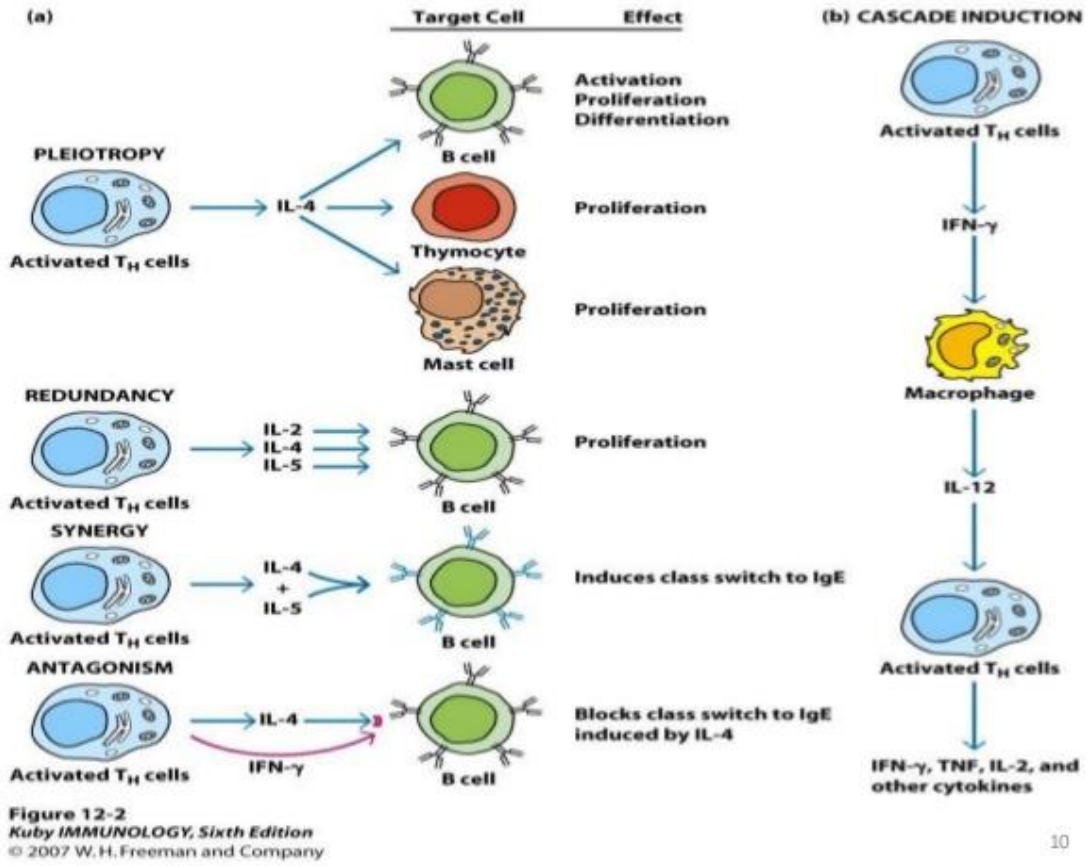
2. High affinity:

- The affinity between cytokines ad their receptor is very high. Because of high affinity, cytokines can mediate biological effects at picomolar concentration.

3. Action:

- a particular cytokine possess one of the following action:
- i) **Autocrine action:** the cytokine may binds with the membrane receptor of same cell that secrete it.
- ii) **Paracrine action:** the cytokine may bind to the receptor on a target cell in close proximity to producer cell.
- iii) **Endocrine action:** cytokine may binds to the target cell in distant part of the body.

4. Attributes:



- cytokines exhibit induction of following attributes:
- i) **Pleiotropy:** a given cytokine has different biological effects on different target cell.
- ii) **Redundancy:** two or more cytokines that mediate similar function.
- iii) **Synergy:** combined effect of two cytokine on cellular activity is greater than effect of individual cytokine.
- iv) **Antagonism:** the effect of one cytokine inhibit the effect of another cytokine.

- v) **Cascade:** when the action of one cytokine on a target cell induce that cell to produce one or more other cytokines, which in turn induce other cell to produce cytokines.

5. Biological functions of cytokines:

- Stimulate development of cellular and humeral immune response
- Induction of inflammatory response
- Regulation of hematopoiesis
- Control of cellular proliferation and differentiation
- Healing of wounds

Cytokines family:

1. Hematopoietin family
2. Chemokines family
3. Interferon family
4. Tumor necrosis factor (TNF) family
5. Colony stimulating factor (CSF) family

Cytokine receptor family:

Cytokines exert their biological function by binding with specific receptor on membrane of target cell. Many types of target cell expresses receptor and are susceptible to cytokine binding.

Cytokine receptor belongs to one of the following family.

1. Type-I cytokine receptor family; Hematopoietin receptor family:

- Majority of cytokine receptor belongs to this class. These receptors have certain conserved motifs in their extracellular amino-acid domain, and lack an intrinsic protein tyrosine kinase activity
- This family includes receptors for IL2 (beta-subunit), IL3, IL4, IL5, IL6, IL7, IL9, IL11, IL12, GM-CSF, G-CSF, Epo, LIF, CNTF, and also the receptors for Thrombopoietin (TPO), Prolactin, and Growth hormone.

- Type I cytokine receptor family is subdivided into three subsets on the basis of the ability of family members to form complexes with one of three different types of receptor signaling components (gp130, common beta, and common gamma – the gamma-chain of the IL2 receptor).

2. Type-II cytokine receptor family; Interferon receptor family:

- There are multimeric receptors composed of heterologous subunits, and are receptors mainly for interferons.
- This family includes receptors for IFN-alpha, IFN-beta, IFN-gamma, IL10, IL22, and tissue factor.

3. Tumor necrosis factor (TNFR) family:

- These receptor family share a cysteine-rich domain (CRD) formed of three disulfide bonds surrounding a core motif of CXXCXXC creating an elongated molecule.
- TNFR is associated with pro-caspases through adapter proteins (FADD, TRADD, etc.) that can cleave other inactive pro-caspases and trigger the caspase cascade, irreversibly committing the cell to apoptosis.

4. Chemokine receptor family:

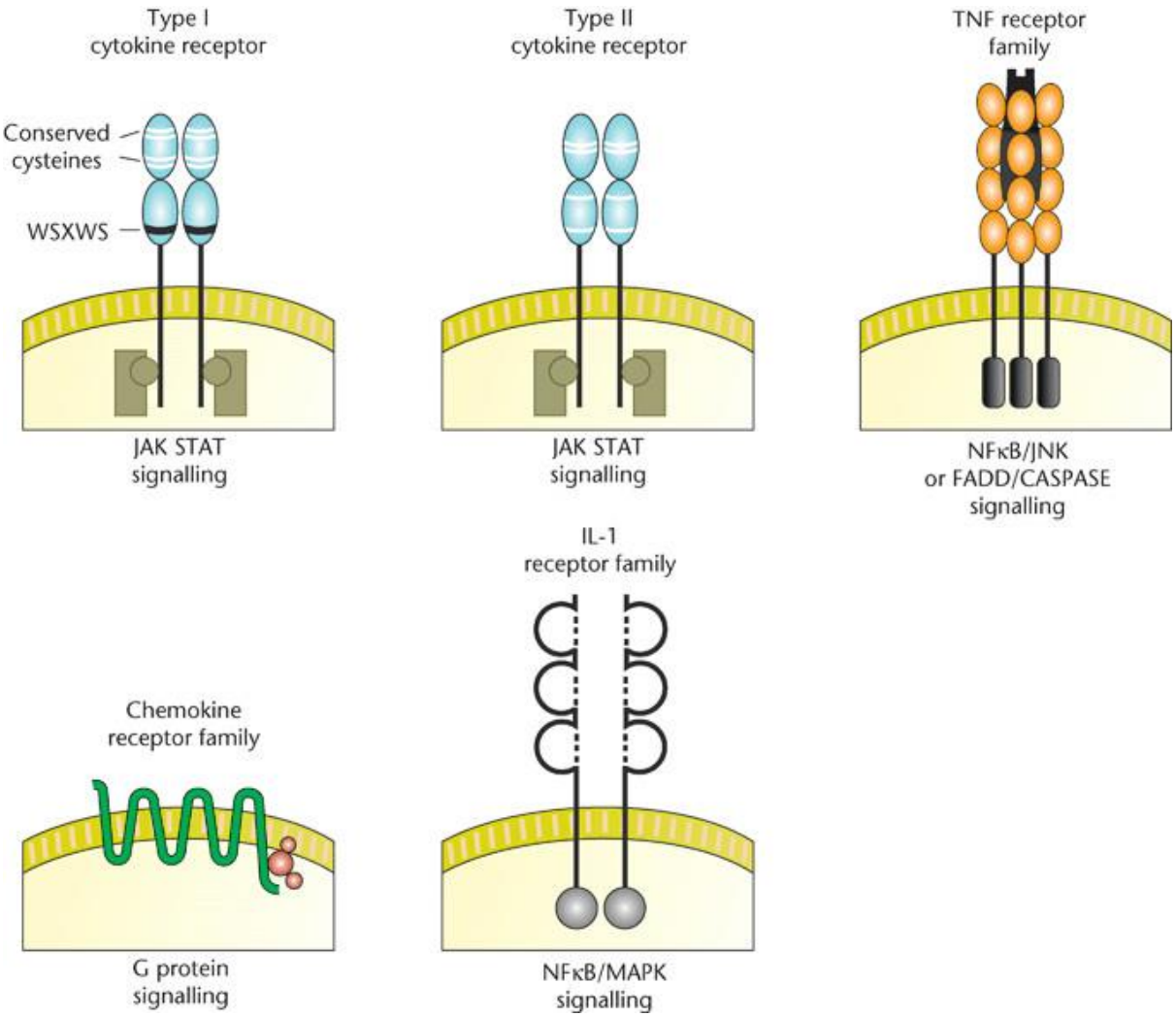
- These receptors are G protein-coupled receptors with 7 transmembrane structure and couple to G-protein for signal transduction.
- Chemokine receptors are divided into different families: CC chemokine receptors, CXC chemokine receptors, CX3C chemokine receptors, and XC chemokine receptor (XCR1).

5. Immunoglobulin super family receptor:

- There induce receptors for IL-1, M-CSF, c-Kit, IL-18. It plays role in inflammation.

6. TGF-beta receptor family:

- These are single pass serine/threonine kinase receptors.
- TGF-beta receptors include TGFBR1, TGFBR2, and TGFBR3 which can be distinguished by their structural and functional properties.



source; researchgate.net

Cytokines: properties and receptors

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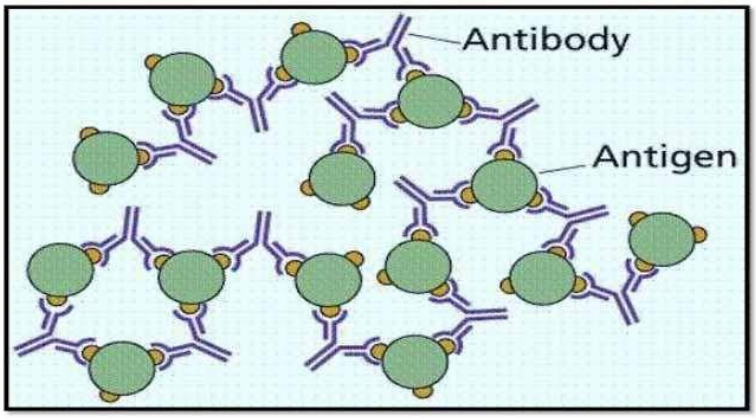
DEPARTMENT OF BIOTECHNOLOGY

UNIT – IV – Immunotechniques – SBTA1402

Antigen-Antibody Interaction

The interactions between antigens and antibodies are known as ***antigen-antibody reactions***. The reactions are highly specific, and an antigen reacts only with antibodies produced by itself or with closely related antigens. Antibodies recognize molecular shapes (epitopes) on antigens. Generally, the better the fit of the epitope (in terms of geometry and chemical character) to the antibody combining site, the more favorablethe interactions that will be formed between the antibody and antigen and the higher the affinity of the antibody for antigen. The affinity of the antibody for the antigen is one of the most important factors in determining antibody efficacy in vivo.



The antigen- antibody interaction is bimolecular irreversible association between antigen and antibody. The association between antigen and antibody includes various non-covalent interactions between epitope (antigenic determinant) and variable region (VH/VL) domain of antibody.

Chemical Bonds Responsible for the Antigen-Antibody Reaction

The interaction between the Ab-binding site and the epitope involves exclusively non- covalent bonds, in a similar manner to that in which proteins bind to their cellular receptors, or enzymes bind to their substrates. The binding is reversible and can be

prevented or dissociated by high ionic strength or extreme pH. The following intermolecular forces are involved in Ag–Ab binding:

1. **Electrostatic bonds:** This result from the attraction between oppositely charged ionic groups of two protein side chains; for example, an ionized amino group ($NH4^{+}$) on a lysine in the Ab, and an ionized carboxyl group (COO–) on an aspartate residue in the Ag.
2. **Hydrogen bonding**: When the Ag and Ab are in very close proximity, relatively weak hydrogen bonds can be formed between hydrophilic groups (e.g., OH and C=O, NH and C=O, and NH and OH groups).
3. **Hydrophobic interactions**: Hydrophobic groups, such as the side chains of valine, leucine, and phenylalanine, tend to associate due to Van der Waals bonding and coalesce in an aqueous environment, excluding water molecules from their surroundings. As a consequence, the distance between them decreases, enhancing the energies of attraction involved. This type of interactionis estimated to contribute up to 50% of the total strength of the Ag–Ab bond.
4. **Van der Waals bonds:** These forces depend upon interactions between the "electron clouds" that surround the Ag and Ab molecules. The interaction has been compared to that which might exist between alternating dipoles in twomolecules, alternating in such a way that, at any given moment, oppositely oriented dipoles will be present in closely apposed areas of the Ag and Ab molecules.

Each of these non-covalent interactions operates over very short distance (generally about 1 Å) so, Ag-Ab interactions depends on very close fit between antigen and antibody.

Strength of Ag-Ab interaction:

1. **Affinity:**

Affinity measures the strength of interaction between an epitope and an antibody's antigen binding site. It is defined by the same basic thermodynamic principles that govern any reversible biomolecular interaction:

$$K_A = \frac{[Ab\text{-}Ag]}{[Ab]\,[Ag]}$$

- **KA** = affinity constant
- **[Ab]** = molar concentration of unoccupied binding sites on the antibody
- **[Ag]** = molar concentration of unoccupied binding sites on the antigen
- **[Ab-Ag]** = molar concentration of the antibody-antigen complex

In other words, KA describes how much antibody-antigen complex exists at the point when equilibrium is reached. The time taken for this to occur depends on rateof diffusion and is similar for every antibody. However, high-affinity antibodies will bind a greater amount of antigen in a shorter period of time than low-affinity antibodies. KA can therefore vary widely for antibodies from below 10^5 mol^{-1} to above 10^{12} mol^{-1}, and can be influenced by factors including pH, temperature and buffer composition.

- Combined strength of total non-covalent interactions between single Ag- bindingsite of Ab and single epitope is affinity of Ab for that epitope.
- Low affinity Ab: Bind Ag weakly and dissociates readily.
- High affinity Ab: Bind Ag tightly and remain bound longer.

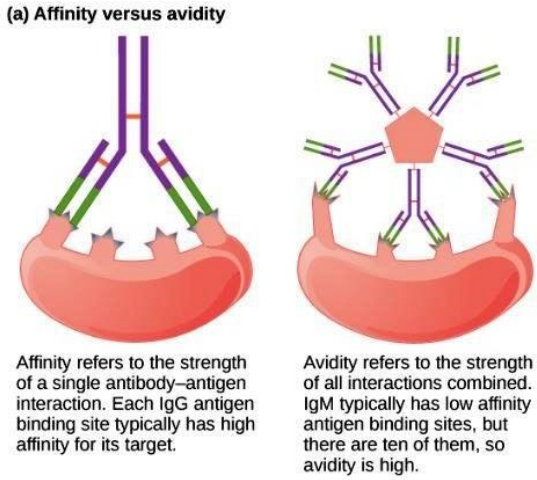
2. Avidity:

Antibodies and antigens are multivalent, meaning they possess more than one binding site. The measure of the total binding strength of an antibody at every binding site is termed avidity. Avidity is also known as the functional affinity.

Avidity is determined by three factors.

- **The binding affinity:** The strength of the relationship at a singular binding site.
- **The valency:** The total number of binding sites involved.
- **The structural arrangement:** The structure of the antigen and antibody involved.
- All antibodies are multivalent e.g. IgGs are bivalent and and IgMs are decavalent. The greater an immunoglobulin's valency (number of antigen binding sites), the greater the amount of antigen it can bind. Similarly, antigens can demonstrate multivalency

because they can bind to more than one antibody. Multimeric interactions between an antibody and an antigen help their stabilization.



- A favorable structural arrangement of antibody and antigen can also lead to a more stable antibody-antigen complex

- Strength of multiple interactions between multivalent Ab and Ag is avidity. Avidity is better measure of binding capacity of antibody than affinity. High avidity can compensate low affinity.

3. **Cross reactivity:**

- Antibody elicited by one Ag can cross react with unrelated Ag if they shareidentical epitope or have similar chemical properties.

Types of Ag-Ab reactions:

1. Agglutination
2. Precipitation
3. Complement Fixation
4. Enzyme linked Immunosorbent Assay
5. RadioImmuno Assay
6. Western Blotting

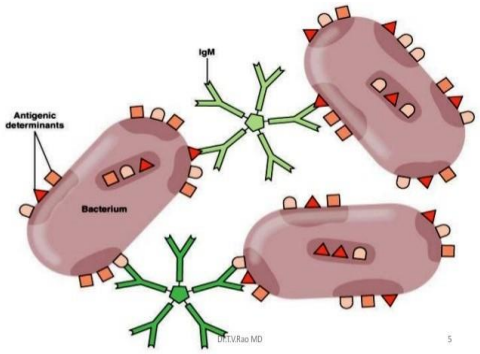
Agglutination

The interaction between **antibody and a particulate antigen** results in visible clumping called agglutination. Antibodies that produce such reactions are called agglutinins. Better agglutination takes place with IgM antibody than with IgG antibodies. Excess of an antibody also inhibits agglutination reaction; this inhibition is called prozone phenomenon.

1. Agglutination is more sensitive than precipitation for the detection of antibodies.
2. Agglutination occurs optimally when antigens and antibodies react in equivalent proportions.

The prozone phenomenon may be seen when either an antibody or an antigen is in excess. Incomplete or monovalent antibodies do not cause agglutination, though they combine with the antigen. They may act as blocking antibodies, inhibiting agglutination by the complete antibody added subsequently.

Types of agglutination



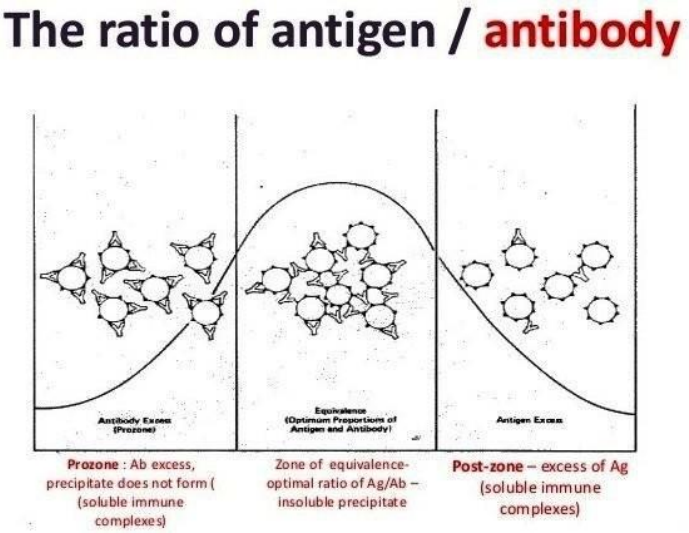
1. Slide agglutination: Serotyping.
2. Tube agglutination: e.g. **Widal test.**
3. Indirect (passive agglutination): where soluble antigens are coated on vehicle particle e.g. latex particle, RBCs.

Application of Agglutination reaction:

1. Cross-matching and grouping of blood.
2. Identification of Bacteria. E.g. Serotyping of *Vibrio cholera*, Serotyping of *Salmonella* Typhi and Paratyphi.
3. Serological diagnosis of various diseases. E.g Rapid plasma regains **(RPR)** test for Syphilis, Antistreptolysin O **(ASO)** test for rheumatic fever.
4. Detection of unknown antigen in various clinical specimens. E.g. detectionof **Vi** antigen of *Salmonella* Typhi in the urine.

Precipitation

It is a type of antigen-antibody reaction, in which the antigen occurs in a soluble form. When a soluble antigen reacts with its specific antibody, at an optimum temperature and P^H in the presence of electrolyte antigen-antibody complex forms insolubleprecipitate. This reaction is called a precipitation reaction. A lattice is formed betweenthe antigens and antibodies; in certain cases, it is visible as an insoluble precipitate. Antibodies that aggregate soluble antigens are called **precipitins.**



The interaction of antibody with soluble antigen may cause the formation of insoluble lattice that will precipitate out of solution. Formation of an antigen-antibody latticedepends on the valency of both the antibody and antigen. The antibody must be **bivalent**; a precipitate will not form with monovalent Fab fragments. The antigen must be **bivalent or polyvalent**; that is it must have at least two copies of same epitopeor different epitopes that react with different antibodies present in polyclonal sera. Antigen and antibody must be in an appropriate concentration relative to each other.

1. Antigen access: Too much antigen prevents efficient crosslinking/latticeformation.
2. Antibody access: Too much antibody prevents efficient crosslinking/lattice formation.
3. Equivalent Antigen and Antibody: Maximum amount of lattice (Precipitate) is formed.

Application of Precipitation reaction:

1. Detection of unknown antibody to diagnose infection e.g. VDRL test for syphilis.
2. Standardization of toxins and antitoxins.
3. Identification of Bacteria e.g. Lancified grouping of streptococci.
4. Identification of bacterial component e.g Ascoli's thermoprecipitin test for *Bacillus anthracis.*

PRECIPITATION REACTION – DEFINITION AND APPLICATIONS

Precipitation definition

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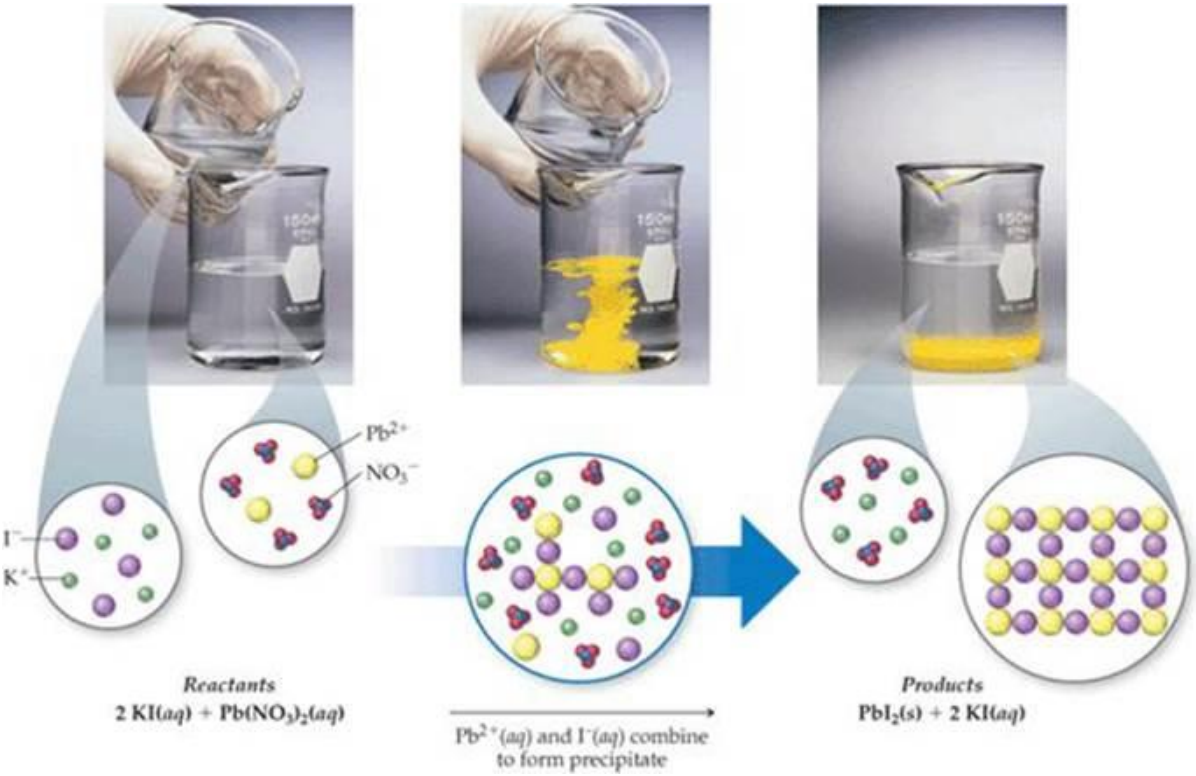

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Prozone phenomenon

Antigen and antibody reaction occurs optimally only when the proportion of the antigen and antibody in the reaction mixture is equivalent. On either side of the equivalence zone, precipitation is actually prevented because of an excess of either antigen or antibody. The zone of antibody excess is known as the ***prozone phenomenon*** and the zone of antigen excess is known as **post zone phenomenon**.

In the prozone phenomenon, there is too much antibody for efficient lattice formation. This is because antigen combines with only a few antibodies and no cross-linkage is formed. In the post zone phenomenon, small aggregates are surrounded by excess antigen and again no lattice network is formed. Thus, for precipitation reactions to be detectable, they must be run in the zone of equivalence. When instead of sedimenting, the precipitate remains suspended as floccules, the reaction is known as ***flocculation***.

Precipitation reactions are based on the interaction of antibodies and antigens. They are based on two soluble reactants that come together to make one insoluble product, the precipitate. These reactions depend on the formation of lattices (cross-links) when antigen and antibody exist in optimal proportions. Excess of either component reduces lattice formation and subsequent precipitation. Precipitation reactions differ from agglutination reactions in the size and solubility of the antigen. Antigens are soluble molecules and larger in size in precipitation reactions. There are several precipitation methods applied in the clinical laboratory for the diagnosis of disease. These can be performed in semi-solid media such as agar or agarose, or non-gel support media such as cellulose acetate.

Applications of the precipitation reaction

1. Detection of unknown antibody to diagnose infection e.g. VDRL test for syphilis.
2. Standardization of toxins and antitoxins.
3. Identification of Bacteria e.g. Lancified grouping of streptococci.
4. Identification of bacterial component e.g Ascoli's thermoprecipitin test for *Bacillus anthracis.*

   I Ouchterlony Double Immunodiffusion technique

   II Radial Immunodiffusion

   III Rocket Immunoelectrophoresis

   IV Immunoelectrophoresis

I Ouchterlony Double Immunodiffusion technique

- Immuno-diffusion is a technique for the detection or measurement of antibodies and antigens by their **precipitation** which involves diffusion through a substance such as agar or gel agarose. Simply, it denotes precipitation in gel.
- It refers to one of the several techniques for obtaining a precipitate between an antibody and its specific antigen.
- Immunodiffusion reactions are classified based on the:

1. Number of reactants diffusing (Single diffusion/Double diffusion)
2. Direction of diffusion (One dimension/Two dimension)

- They thus may be of the following types:

1. Single diffusion in one dimension
2. Single diffusion in two dimensions
3. Double diffusion in one dimension
4. Double diffusion in two dimensions

Double Immuno-diffusion

- Double immunodiffusion is an agar gel immunodiffusion.
- It is a special precipitation reaction on gels where antibodies react with specific antigens forming large antigen-antibody complexes which can be observed as a line of the precipitate.
- In double immunodiffusion, both the antibody and antigen are allowed to diffuse into the gel.
- After application of the reactants in their respective compartments, the antigen and the antibody diffuse toward each other in the common gel and a precipitate is formed at the place of equivalence.

Double diffusion in one dimension

The method also called Oakley–Fulthrope procedure involves the incorporation of the antibody in agar gel in a test tube, above which a layer of plain agar is placed. The antigen is then layered on top of this plain agar. During incubation, the antigen and antibody move toward each other through the intervening layer of plain agar. In this zone of plain agar, both antigen and antibody react with each other to form a band of precipitation at their optimum concentration.

Double diffusion in two dimensions

It is more commonly known as Ouchterlony double diffusion or passive double immunodiffusion. In this method, both the antigen and antibody diffuse independently through agar gel in two dimensions, horizontally and vertically.

Objectives

The Ouchterlony double immunodiffusion test may be carried out with one or more of the following objectives:

1. To detect antigen-antibody complexes.
2. Describe the circumstances under which antigen-antibody complexes precipitate out.
3. Detect the presence of an antigen-specific antibody.
4. To test the similarity between antigens.

Principle

In the test, an antigen solution or a sample extract of interest is placed in wells bore on gel plates while sera or purified antibodies are placed in other remaining wells (Mostly, an antibody well is placed centrally). On incubation, both the antigens in the solution and the antibodies each diffuse out of their respective wells. In case of the antibodies recognizing the antigens, they interact together to form visible immune complexes which precipitate in the gel to give a thin white line (precipitin line) indicating a reaction.

In case multiple wells are filled with different antigen mixtures and antibodies, the precipitate developed between two specific wells indicate the corresponding pair of antigen-antibodies.

Materials Required

Glass plate or Petri plate, Agarose, Gel borer, Buffer, Antiserum, Antigen solutions

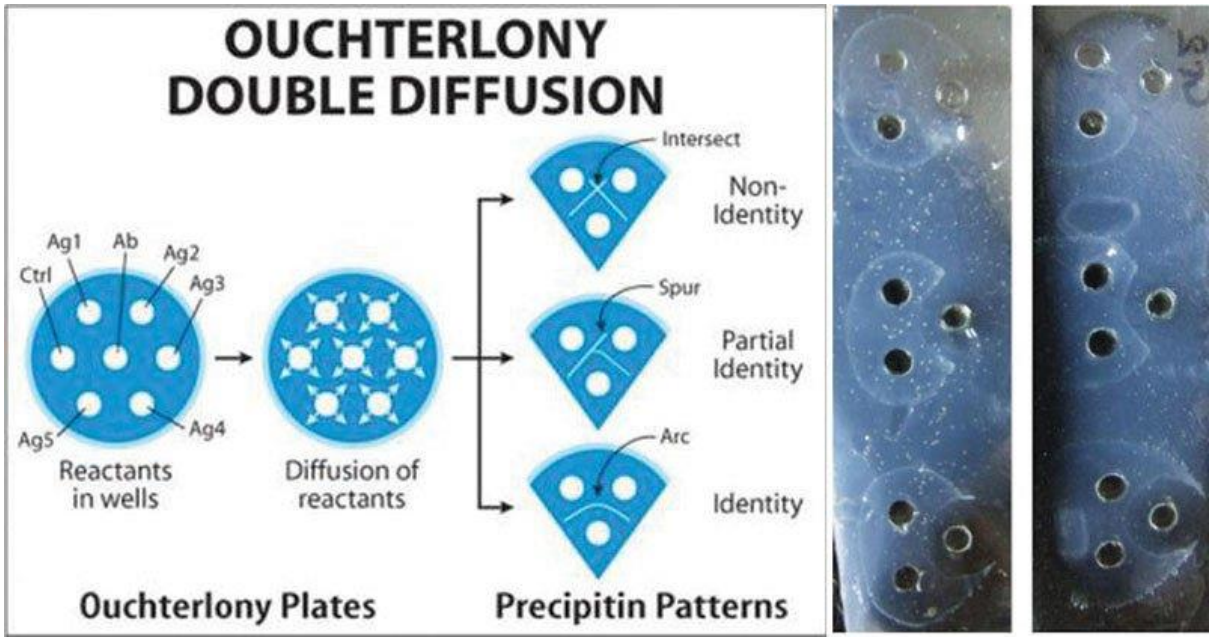
Procedure

1. Dissolve 100 mg of agarose in 10 ml of the buffer by boiling to completely dissolve the agarose.
2. Cool solution to 55 °C and pour agarose solution to a depth of 1 – 2 mm on a clean glass plate (petri dish or rectangular plate) placed on a horizontal surface.
3. Allow the gel to set for 30 minutes.
4. Wells are punched into the gel using a gel borer corresponding to the marks on the template if used.

5. Fill wells with solutions of antigen and antiserum (of same or different dilutions) until the meniscus just disappears. Antiserum is usually placed in the central well and different antigens are added to the wells surrounding the center well.
6. Incubate the glass plate in a moist chamber overnight at 37 °C.

Results

- The presence of an opaque precipitant line between the antiserum and antigen wells indicates antigen-antibody interaction.
- Absence of precipitant line suggests the absence of reaction.
- When more than one well is used there are many possible outcomes based on the reactivity of the antigen and antibody selected.



- The results may be either of the following:
    - **A full identity (i.e. a continuous line):** Line of precipitation at their junction forming an arc represents serologic identity or the presence of a common epitope in antigens.
    - **Non-identity (i.e. the two lines cross completely):** A pattern of crossed lines demonstrates two separate reactions and indicates that the compared antigens are unrelated and share no common epitopes.
    - **Partial identity (i.e. a continuous line with a spur at one end):** The two antigens share a common epitope, but some antibody molecules are not captured by the antigen and traverse through the initial precipitin line to combine with additional epitopes found in the more complex antigen.

- The pattern of the lines that form can determine whether the antigens are the same.

Applications

1. It is useful for the analysis of antigens and antibodies.
2. It is used in the detection, identification, and quantification of antibodies and antigens, such as immunoglobulins and extractable nuclear antigens.
3. Agar gel immunodiffusions are used as serologic tests that historically have been reported to identify antibodies to various pathogenic organisms such as *Blastomyces*.
4. Demonstration of antibodies in serodiagnosis of smallpox.
5. Identification of fungal antigens.
6. Elek's precipitation test in the gel is a special test used for demonstration of toxigenicity of *Corynebacterium diphtheriae*.

II Radial Immunodiffusion- Objectives, Principle, Procedure, Results, Applications, Advantages and Limitations

Immuno-diffusion is a technique for the detection or measurement of antibodies and antigens by their precipitation which involves diffusion through a substance such as agar or gel agarose. Simply, it denotes precipitation in gel.

It refers to any of the several techniques for obtaining a precipitate between an antibody and its specific antigen.

This can be achieved by:

1. a) Suspending antigen/antibody in a gel and letting the other migrate through it from a well or,
2. b) Letting both antibody and antigen migrate through the gel from separate wells such that they form an area of precipitation.

Based on the method employed, immuno-diffusion may be:

1. **Radial immunodiffusion**
2. **Ouchterlony Double Diffusion**

Radial immunodiffusion (RID) or **Mancini method** is also known as **Mancini immunodiffusion** or **single radial immunodiffusion assay**. It is a single diffusion technique whereby a solution containing the antigen is placed into wells in a gel or agar surface evenly impregnated with antibody. The diameter of the ring that precipitates around the well as a result of antigen antibody reaction corresponds to the amount of antigen in the solution.

Objectives of Radial Immunodiffusion

The Mancini immunodiffusion test may be carried out with one or more of the following objectives:

1. To detect antigen-antibody complexes.
2. Describe the circumstances under which antigen-antibody complexes precipitate out.
3. Determine relative concentration of antigens.

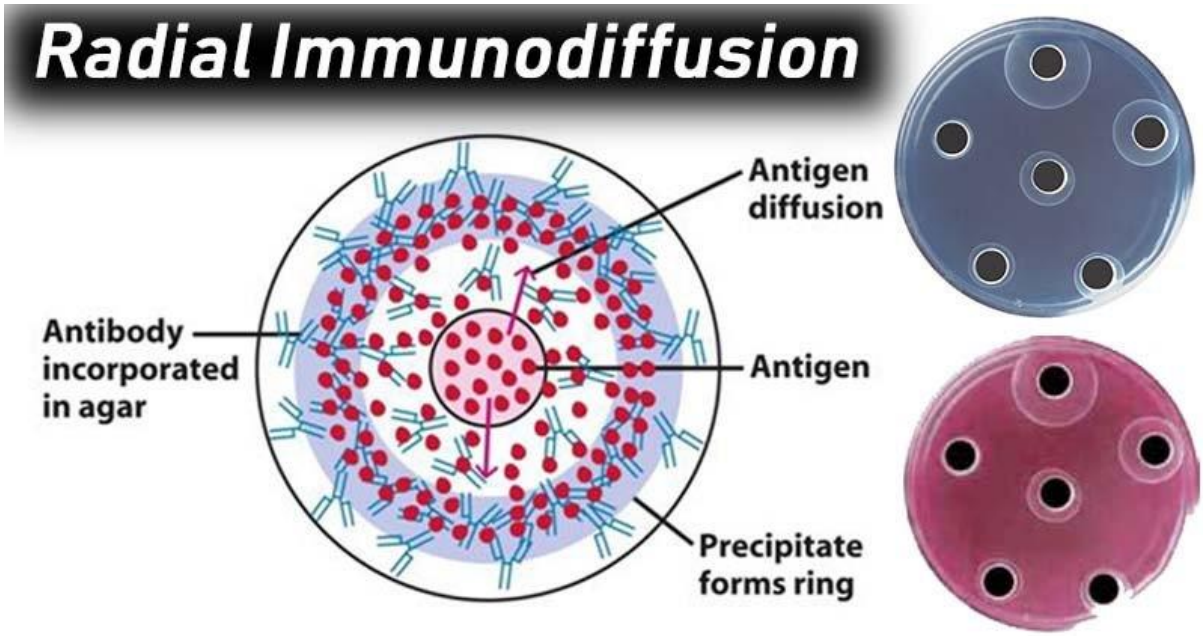
Principle of Radial Immunodiffusion

Radial immuno-diffusion is a type of precipitation reaction. It is thus based on the principles of the precipitin curve which states that antigen-antibody interact forming visible cross-linked precipitate when the proper ratio of antigen to antibody is present. In the test, antibody is incorporated into agar and poured into a glass plate to form a uniform layer. Circular wells are cut into the agar and antigen is introduced into the wells. Specific antigens to the impregnated antibodies diffuse through the agar in all directions from the well and react with the antibody present forming visible precipitate or a precipitin ring. Ring shaped bands of precipitates from concentrically around the well indicating reaction. The diameter of the precipitate ring formed, corresponds to the amount of antigen in the solution.

Procedure of Radial Immunodiffusion

1. An agar containing an appropriate antiserum (antibody) is poured in plates.
2. Carefully circular wells are cut and removed from the plates.
3. A single or series of standards containing known concentration of antigen are placed in separate wells, while control and "unknown" samples are placed in other remaining wells.
4. As the antigen diffuses radially, a ring of precipitate will form in the area of optimal antigen – antibody concentration.
5. The ring diameters are measured and noted.
6. A standard curve is prepared using the ring diameters of the standards versus their concentrations. This curve is then used to determine the concentration of the control and unknown samples.

Result Interpretation of Radial Immunodiffusion



1. The presence of a precipitin ring around the antigen wells indicate specific antigen-antibody interaction.
2. Absence of precipitin ring suggest absence of reaction.
3. The greater the amount of antigen in the well, the farther the ring will form from the well.

Applications of Radial Immunodiffusion

- Immuno-diffusion techniques are mostly used in immunology to determine the quantity or concentration of an antigen in a sample.
- Estimation of the immunoglobulin classes in sera.
- Estimation of IgG, IgM antibodies in sera to influenza viruses.

Other applications include:

- To determine relative concentrations of antibodies in serum.
- Estimate serum transferrin and alpha-feroprotein.
- To compare properties of two different antigens.
- To determine the relative purity of an antigen preparation
- For disease diagnosis
- Serological surveys

Advantages of Radial Immunodiffusion

1. Precipitation in gels is believed to provide more specific and sensitive results than other methods available.
2. The reaction is in the form of bands of precipitation and can be stained for better viewing as well as preservation.

3. If a large number of antigens are present, each antigen-antibody reaction will give rise to a separate line of precipitation.
4. This technique also indicates identity, cross reaction and non identity between different antigens.

Limitations of Radial Immunodiffusion

1. Long reaction time (18-48 hours)
2. It has also been proposed that the results of Mancini's test is influenced by the presence bound metal cations in the test samples (protein).
3. Single diffusion menthod of precipitation is considered relatively wasteful than other methods.
4. The test has been recently replaced by more sensitive and automated methods, such as nephelometry and enzyme-linked immunosorbent assays.

III Rocket Immunoelectrophoresis-

Objectives, Principle, Procedure, Results, Applications, Advantages and Limitations

- **Rocket Immunoelectrophoresis** is an adaptation of radial immunodiffusion developed by Laurell. It is also known as electroimmunoassay or electroimmunodiffusion.
- It is called as "rocket electrophoresis" due to the appearance of the precipitin bands in the shape of cone-like structures (rocket appearance) at the end of the reaction.
- In rocket immunoelectrophoresis, antigen migrates in an electric field in a layer of agarose containing an appropriate antibody.
- The migration of the antigen toward the anode gives rise to rocket-shaped patterns of precipitation. The area under the rocket is proportional to antigen concentration.

Objectives of Rocket Immunoelectrophoresis

1. To detect antigen-antibody complexes.
2. Determine the concentration of antigen in an unknown sample.

Principle of Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis is a quantitative one-dimensional single electro-immunodiffusion technique. In this method antibody is incorporated in the gel at a pH value at which the antibodies remain essentially immobile. Antigen is placed in wells cut in the gel. Electric current is then passed

through the gel, which facilitates the migration of negatively charged antigens into the agar. As the antigen moves out of the well and enters the agarose gel, it combines with the antibody to form immune complex which becomes visible. During the initial phase there is considerable antigen excess over antibody and no visible precipitation occurs. However, as the antigen sample migrates further through the agarose gel, more antibody molecules are encountered that interact with the antigen to form immune complex. This results in formation of a precipitin line that is conical in shape, resembling a rocket.

The greater the amount of antigen loaded in a well, the further the antigen will have to travel through the gel before it can interact with sufficient antibody to form a precipitate. Thus, the height of the rocket, measured from the well to the apex and area are directly proportional to the amount of antigen in the sample.

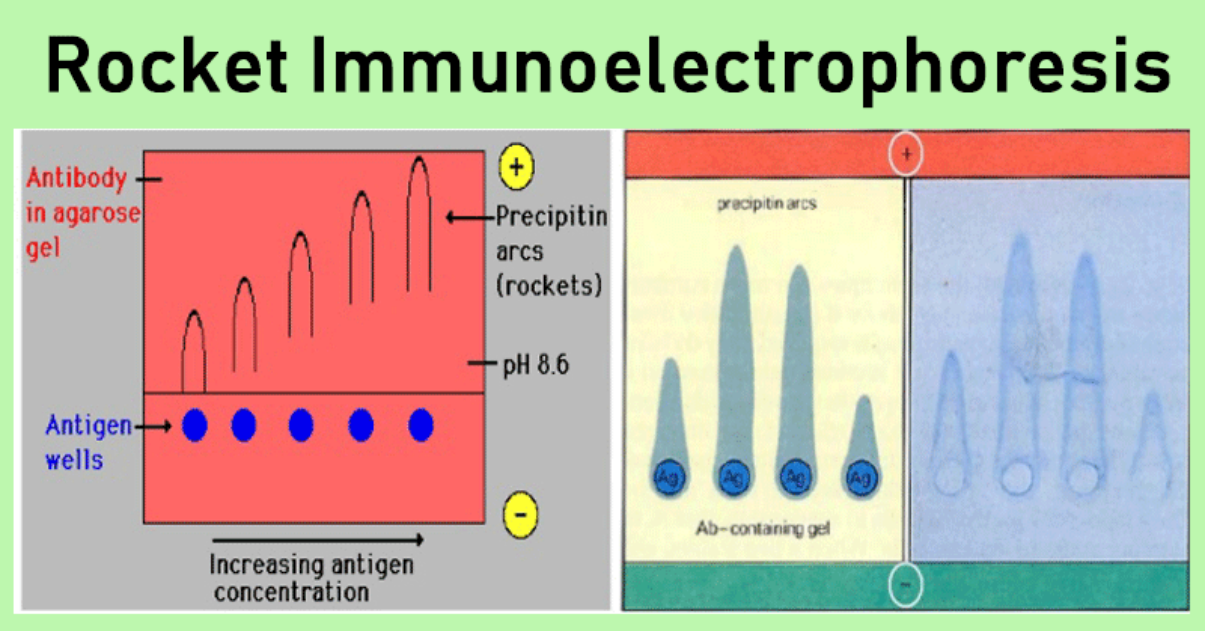
Materials Required for Rocket Immunoelectrophoresis

Agarose, Antigen, Antiserum, Assay Buffer, Electrophoresis apparatus, Glass slides

Procedure of Rocket Immunoelectrophoresis

1. About 15 ml of 1 % agarose gel is prepared.
2. The solution is cooled to 55-60^{o}C and 250 µl of antiserum added to 13 ml of agarose solution. It is well mixed for uniform distribution of antibodies.
3. Agarose solution containing the antiserum is poured onto to grease-free glass plate placed on a horizontal surface and the gel is allowed to set for 30 minutes.
4. The glass plate is on the template and wells punched with the help of a gel puncher.
5. 10 µl of the standard antigen and test antigen samples are added to the wells.
6. 1X TBE buffer is poured into the electrophoresis tank such that it just covers the gel.
7. Electrophoresis is carried out at 80-120 volts and 60-70 mA until the antigen travels 3-4 cms from the well.
8. The glass plate is incubated in a moist chamber overnight at 37^{o} C and the results interpreted.
9. In case positive for reaction, the tips of the precipitin peaks are marked and the peak height measured from the upper edge of the well to the tip of the peak.
10. A graph is plotted of the rocket height (on Y-axis) versus the concentration of antigen (on X-axis) on a semi-log graph sheet. The concentration of the unknown is determined from the graph by finding the concentration against the rocket height.

Result Interpretation of Rocket Immunoelectrophoresis



- A precipitation 'rocket' spreading out from the loading well indicate positive reaction or specific antigen-antibody reaction due to the presence of antibody specific to the antigen.
- The absence of the precipitation indicates no reaction or the absence of any corresponding antibody – antigen.
- The height of the rocket, and its area are directly proportional to the amount of antigen in the sample, that is, the height of the precipitin peak depends on the concentration of antigens loaded in the corresponding wells.

Applications of Rocket Immunoelectrophoresis

1. Rocket electrophoresis is used mainly for quantitative estimation of antigen in the serum.
2. The method has been used for quantization of human serum proteins before automated methods became available.
3. Determining the concentration of a specific protein in a protein mixture.
4. In estimation of immunoglobulin protease activity.
5. Studies dealing with antigenic relationships between organisms.
6. In enzyme activity electrophoresis.

Advantages of Rocket Immunoelectrophoresis

- Simple, quick, and reproducible method.
- Several unknown samples can be analyzed on a single plate.
- Concentrations of proteins as little as 1 µg/mL can be measured requiring as little as 20 ng of protein to be loaded in a well.

Limitations of Rocket Immunoelectrophoresis

- These techniques allow quantitative analysis of antigens, but are not applicable to complex mixtures.

Related Techniques

Fused rocket immunoelectrophoresis is a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.

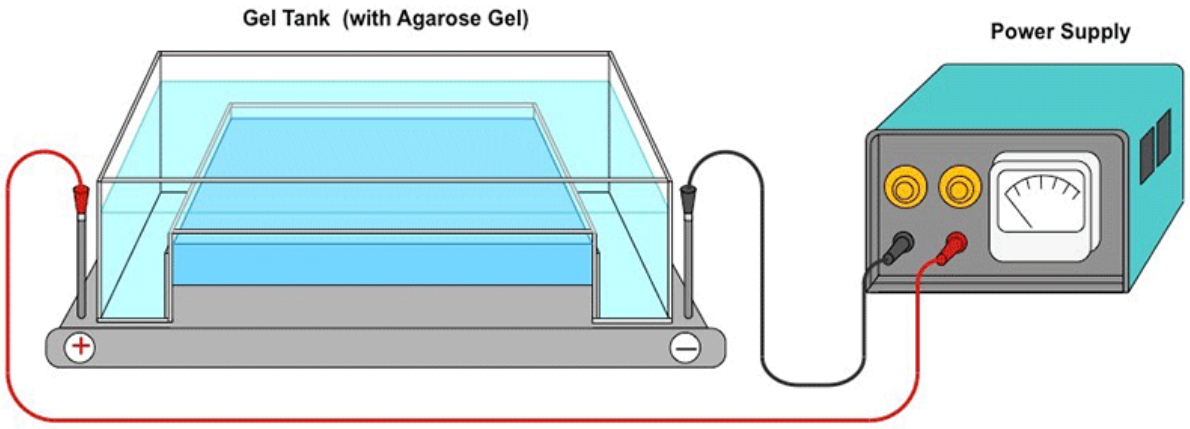
Two-dimensional immunoelectrophoresis is a variant of rocket electrophoresis. The test is a two-stage procedure. In the first stage, antigens in solution are separated by electrophoresis. In the second stage, electrophoresis is carried out again, but perpendicular to that of first stage to obtain rocket-like precipitation.

IV Immunoelectrophoresis-

Principle, Procedure, Results and Applications, Advantages and Limitations

- Immunoelectrophoresis refers to precipitation in agar under an electric field.
- It is a process of a combination of immuno-diffusion and electrophoresis.
- An antigen mixture is first separated into its component parts by electrophoresis and then tested by double immuno-diffusion.
- Antigens are placed into wells cut in a gel (without antibody) and electrophoresed. A trough is then cut in the gel into which antibodies are placed.
- The antibodies diffuse laterally to meet diffusing antigens, and lattice formation and precipitation occur permitting determination of the nature of the antigens.
- The term “immunoelectrophoresis” was first coined by Grabar and Williams in 1953.

Immunoelectrophoresis



Principle of Immunoelectrophoresis

When an electric current is applied to a slide layered with gel, the antigen mixture placed in wells is separated into individual antigen components according to their charge and size. Following electrophoresis, the separated antigens are reacted with specific antisera placed in troughs parallel to the electrophoretic migration and diffusion is allowed to occur. Antiserum present in the trough moves toward the antigen components resulting in the formation of separate precipitin lines in 18-24 hrs, each indicating reaction between individual proteins with its antibody.

Procedure of Immunoelectrophoresis

1. Agarose gel is prepared on a glass slide put in a horizontal position.
2. Using the sample template, wells are borne on the application zone carefully.
3. The sample is diluted 2:3 with protein diluent solution (20µl antigen solution +10 µl diluent).
4. Using a 5 µl pipette, 5 µl of control and sample is applied across each corresponding slit (Control slit and Sample slit).
5. The gel is placed into the electrophoresis chamber with the samples on the cathodic side, and electrophoresis runs for 20 mins/ 100 volts.
6. After electrophoresis completes, 20 µl of the corresponding antiserum is added to troughs in a moist chamber and incubated for 18- 20 hours at room temperature in a horizontal position.
7. The agarose gel is placed on a horizontal position and dried with blotter sheets.

8. The gel in saline solution is soaked for 10 minutes and the drying and washing repeated twice again.
9. The gel is dried at a temperature less than 70°C and may be stained with protein staining solution for about 3 minutes followed by decolorizing the gel for 5 minutes in distaining solution baths.
10. The gel is dried and results evaluated.

Results of Immunoelectrophoresis

1. The presence of elliptical precipitin arcs represents antigen-antibody interaction.
2. The absence of the formation of precipitate suggests no reaction.
3. Different antigens (proteins) can be identified based on the intensity, shape, and position of the precipitation lines.

Applications of Immunoelectrophoresis

1. The test helps in the identification and approximate quantization of various proteins present in the serum. Immunoelectrophoresis created a breakthrough in protein identification and in immunology.
2. Immunoelectrophoresis is used in patients with suspected monoclonal and polyclonal gammopathies.
3. The method is used to detect normal as well as abnormal proteins, such as myeloma proteins in human serum.
4. Used to analyze complex protein mixtures containing different antigens.
5. The medical diagnostic use is of value where certain proteins are suspected of being absent (e.g., hypogammaglobulinemia) or overproduced (e.g., multiple myeloma).
6. This method is useful to monitor antigen and antigen-antibody purity and to identify a single antigen in a mixture of antigens.
7. Immunoelectrophoresis is an older method for qualitative analysis of M-proteins in serum and urine.
8. Immunoelectrophoresis aids in the diagnosis and evaluation of the therapeutic response in many disease states affecting the immune system.

Advantages of Immunoelectrophoresis

1. Immunoelectrophoresis is a powerful analytical technique with high resolving power as it combines the separation of antigens by electrophoresis with immunodiffusion against an antiserum.
2. The main advantage of immunoelectrophoresis is that a number of antigens can be identified in serum.

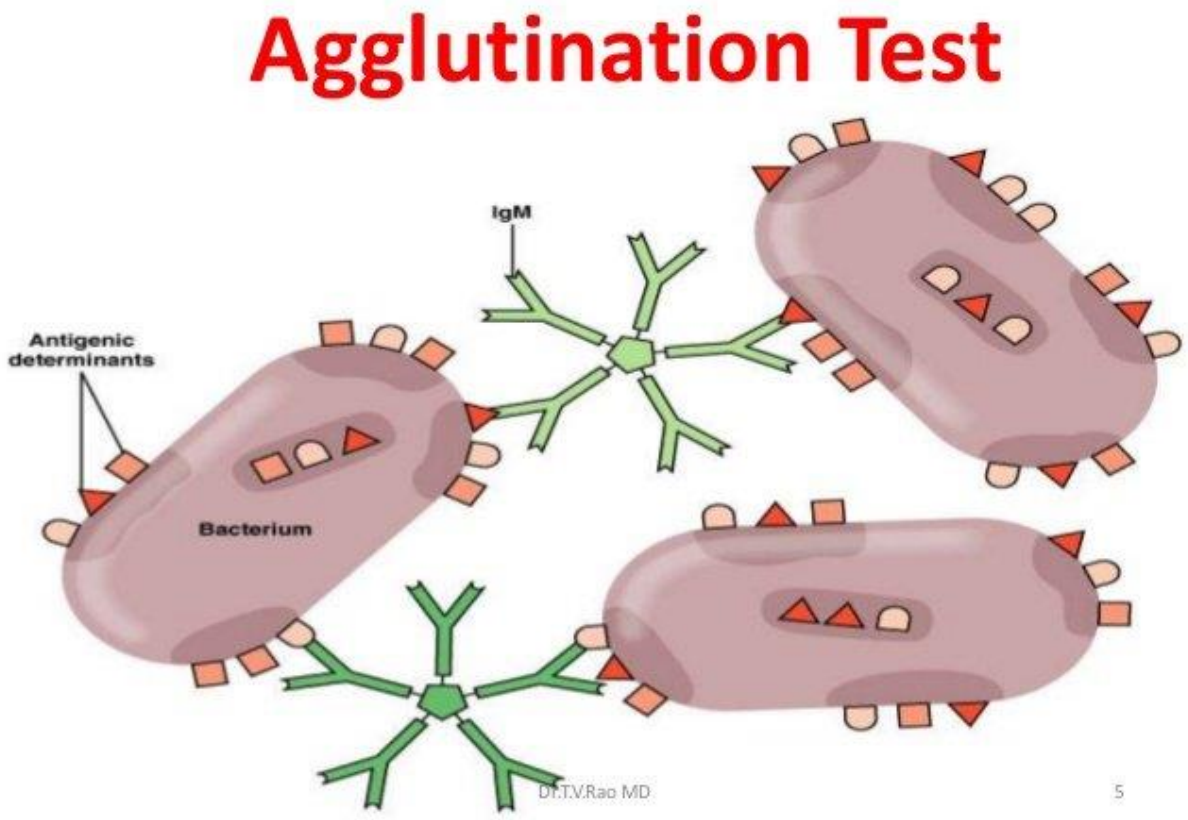
Limitations of Immunoelectrophoresis

1. Immunoelectrophoresis is slower, less sensitive, and more difficult to interpret than Immunofixation electrophoresis.
2. IEP fails to detect some small monoclonal M-proteins because the most rapidly migrating immunoglobulins present in the highest concentrations may obscure the presence of small M-proteins.
3. The use of immunoelectrophoresis in food analysis is limited by the availability of specific antibodies.

AGGLUTINATION – DEFINITION, REACTIONS AND APPLICATIONS

Agglutination definition

Agglutination is an antigen-antibody reaction in which a particulate antigen combines with its antibody in the presence of electrolytes at a specified temperature and pH resulting in the formation of visible clumping of particles. It occurs optimally when antigens and antibodies react in equivalent proportions. This reaction is analogous to the precipitation reaction in that antibodies act as a bridge to form a lattice network of antibodies and the cells that carry the antigen on their surface. Because cells are so much larger than a soluble antigen, the result is more visible when the cells aggregate into clumps.



When particulate antigens react with specific antibody, antigen-antibody complex forms visible clumping under optimum P^H and temperature. Such a reaction is called agglutination. Antibodies that produce such reactions are called **agglutinins**.

What is agglutination?

Agglutination is the visible expression of the aggregation of antigens and antibodies. Agglutination reactions apply to particulate test antigens that have been conjugated to a carrier. The carrier could

be artificial (such as latex or charcoal particles) or biological (such as red blood cells). These conjugated particles are reacted with patient serum presumably containing antibodies. The endpoint of the test is the observation of clumps resulting from that antigen-antibody complex formation. The quality of the result is determined by the time of incubation with the antibody source, amount and avidity of the antigen conjugated to the carrier, and conditions of the test environment (e.g., pH and protein concentration). Various methods of agglutination are used in diagnostic immunology and these include latex agglutination, flocculation tests, direct bacterial agglutination, and hemagglutination.

Agglutination differs from precipitation reaction in that since agglutination reaction takes place at the surface of the particle involved, the antigen must be exposed and be able to bind with the antibody to produce visible clumps. In agglutination reactions, serial dilutions of the antibody solution are made and a constant amount of particulate antigen is added to serially diluted antibody solutions. After several hours of incubation at 37°C, clumping is recorded by visual inspection. The titer of the antiserum is recorded as the reciprocal of the highest dilution that causes clumping. Since the cells have many antigenic determinants on their surface, the phenomenon of antibody excess is rarely encountered.

Prozone phenomenon

The condition of excess antibody, however, is called a **prozone phenomenon.** At a high concentration of antibody, the number of epitopes are outnumbered by antigen-binding sites. This results in the univalent binding of antigen by antibody rather than multivalently and thus, interferes in the crosslinking of antigen (Lattice formation).

Occasionally, antibodies are formed that react with the antigenic determinants of a cell but does not cause any agglutination. They inhibit the agglutination by the complete antibodies added subsequently. Such antibodies are called **blocking antibodies**. Anti-Rh antibodies and anti-brucella antibodies are few examples of such blocking antibodies.

Agglutination tests are easy to perform and in some cases are the most sensitive tests currently available. These tests have a wide range of applications in the clinical diagnosis of non- infectious immune disorders and infectious diseases. Agglutination reactions have a wide variety of applications in the detection of both antigens and antibodies in serum and other body fluids. They are very sensitive and the result of the test can be read visually with ease.

Applications of Agglutination Reactions

1. Cross-matching and grouping of blood.

2. Identification of Bacteria. E.g. Serotyping of *Vibrio cholera*, Serotyping of *Salmonella* Typhi and Paratyphi.
3. Serological diagnosis of various diseases. E.g Rapid plasma regains **(RPR)** test for Syphilis, Antistreptolysin O **(ASO)** test for rheumatic fever.
4. Detection of unknown antigen in various clinical specimens. E.g. detection of **Vi** antigen of *Salmonella* Typhi in the urine.

Types of Agglutination Reactions

Agglutination reactions can be broadly divided into three groups:

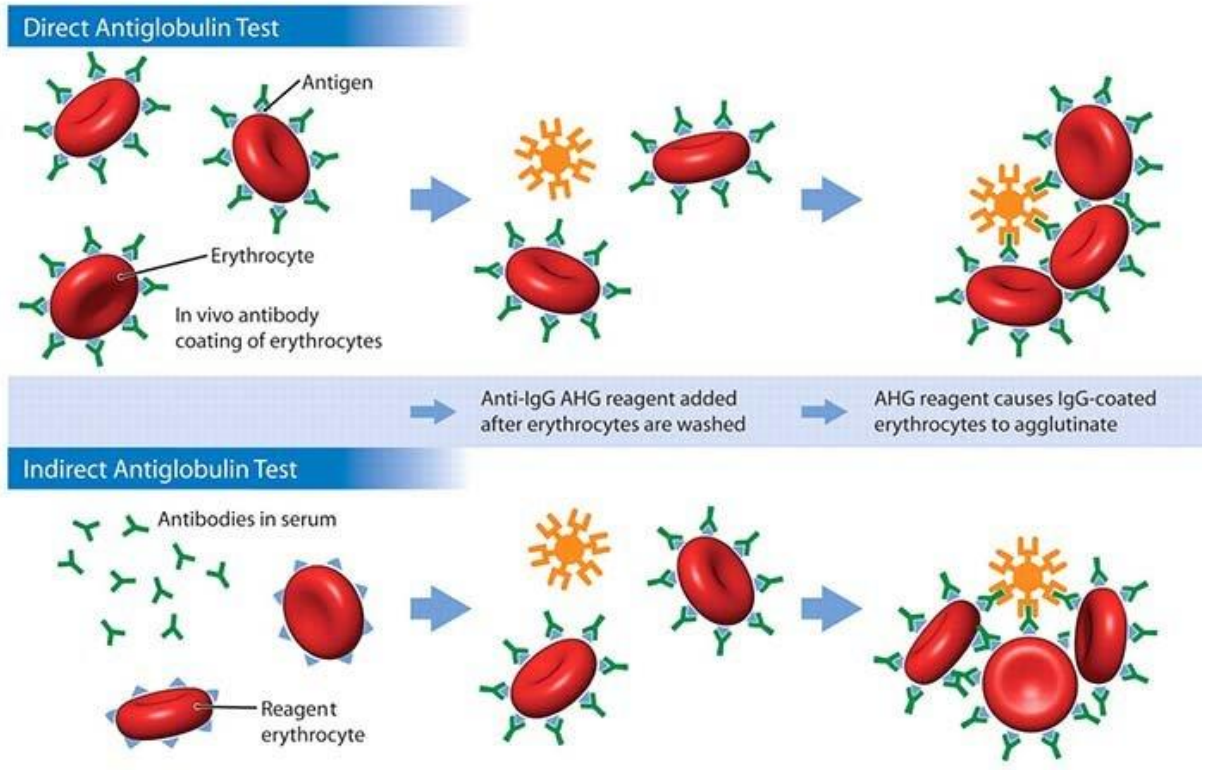
1. Active/Direct agglutination
2. Passive agglutination
3. Hemagglutination

1. Active agglutination

Agglutination reactions where the antigens are found naturally on a particle are known as direct agglutination. In active agglutination, direct agglutination of particulate antigen with specific antibody occurs. Direct bacterial agglutination uses whole pathogens as a source of antigen. It measures the antibody level produced by a host infected with that pathogen. The binding of antibodies to surface antigens on the bacteria results in visible clumps Active agglutination can be of following types:

1. **Slide/Tile agglutination:** Basic type of agglutination reaction that is performed on a slide. Identification of bacterial types represents a classic example of a slide agglutination. In this method suspension of unknown antigen is kept on slide and a drop of standardized antiserum is added or vice versa. A positive reaction is indicated by formation of visible clumps. E.g. Widal test, RPR test.
2. **Tube agglutination:** It is agglutination test performed in tube and standard quantitative technique for determination of antibody titre. In this method serum is diluted in a series of tubes and standard antigen suspensions (specific for the suspected disease) are added to it. After incubation, antigen-antibody reaction is indicated visible clumps of agglutination.
3. **Heterophile agglutination test:** This test depends on demonstration of heterophilic antibodies in serum present in certain bacterial infections.
4. **Antiglobulin (Coombs) test:** This' test was devised by Coombs, Mourant, and Race for detection of incomplete anti-Rh antibodies that do not agglutinate Rh+ erythrocytes in

saline. When serum containing incomplete anti-Rh antibodies is mixed with Rh+ erythrocytes in saline, incomplete antibody antiglobulin coats the surface of erythrocytes but does not cause any agglutination. When such erythrocytes are treated with antiglobulin or Coombs serum (rabbit antiserum against human gamma globulin), then the cells are agglutinated. Coombs test can be direct as well as indirect**.**



In **direct method**, the sensitization of red blood cells (RBCs) with incomplete antibodies takes place *in vivo*. Cell-bound antibodies can be detected by this test in which antiserum against human immunoglobulin is used to agglutinate patient's RBC. In **indirect method**, the sensitization of RBCs with incomplete antibodies takes place *in vitro.* Patient's serum is mixed with normal red cells and antiserum to human immunoglobulin. Agglutination occurs if antibodies are present in serum. **Coombs test is used for detection of anti-Rh antibodies and incomplete antibodies in brucellosis and other diseases**.

2. Passive Agglutination

Passive agglutination employs carrier particles that are coated with soluble antigens. In this either antibody or antigen is attached to certain inert carrier thereby, particles or cells gets agglutinated when corresponding antigen or antibody reacts. Latex particles, Carbon particles, Bantonite etc. are used as inert carriers. E.g. Antigens coated in latex particles used in ASO test. When the antibody

instead of antigens is adsorbed on the carrier particle for detection of antigens, it is called **reverse passive agglutination.**

1. **Latex Agglutination:** It employs latex particles as carrier of antigen or antibodies. In latex agglutination, many antibody or antigen molecules are bound to latex beads (particles), which increases the number of antigen-binding sites. If corresponding antigen or antibody is present in a test specimen, antigen antibody bind and form visible, cross-linked aggregates. Latex agglutination can also be performed with the antigen conjugated to the beads for testing the presence of antibodies in a serum specimen.

3. Hemagglutination test

RBCs are used as carrier particles in hemagglutination tests. RBCs of sheep, human, chick, etc. are commonly used in the test. When RBCs are coated with antigen to detect antibodies in the serum, the test is called **indirect hemagglutination (IHA) test**. Hemagglutination uses erythrocytes as the biological carriers of bacterial antigens, and purified polysaccharides or proteins for determining the presence of corresponding antibodies in a specimen. When antibodies are attached to the RBCs to detect microbial antigen, it is known as **reverse passive hemagglutination (RPHA)**.

Viral hemagglutination: Many viruses including influenza, mumps, and measles have the ability to agglutinate RBCs without antigen–antibody reactions. This process is called viral hemagglutination. This hemagglutination can be inhibited by antibody specifically directed against the virus, and this phenomenon is called **hemagglutination inhibition**.

Coagglutination test: Coagglutination is a type of agglutination reaction in which Cowan I strain of *S. aureus* is used as carrier particle to coat antibodies. Cowan I strain of *S. aureus* contains protein A, an anti-antibody, that combines with the Fc portion of immunoglobulin, IgG, leaving the Fab region free to react with the antigen present in the specimens. In a positive test, protein A bearing *S. aureus* coated with antibodies will be agglutinated if mixed with specific antigen. The advantage of the test is that these particles show greater stability than latex particles and are more refractory to changes in ionic strength.

Uses of Coagglutination test

1. Detection of cryptococcal antigen in the CSF for diagnosis of cryptococcal meningitis;
2. Detection of amoebic and hydatid antigens in the serum for diagnosis of amoebiasis and cystic echinococcosis,
3. Grouping of streptococci and mycobacteria and for typing of *Neisseria gonorrhoeae.*

Coombs Test- Principle, Types, Procedure and Result Interpretation

Coombs test is also known as antiglobulin test. The Coombs test tests for antibodies that may stick to the red blood cells and cause red blood cells to die too early. It was discovered by Coombs, Mourant and Race in 1945. Coombs reagent is antihuman globulin. It is made by injecting human globulin into animals, which produce polyclonal antibodies specific for human immunoglobulins and human complement system factors.

Principle of Coombs test

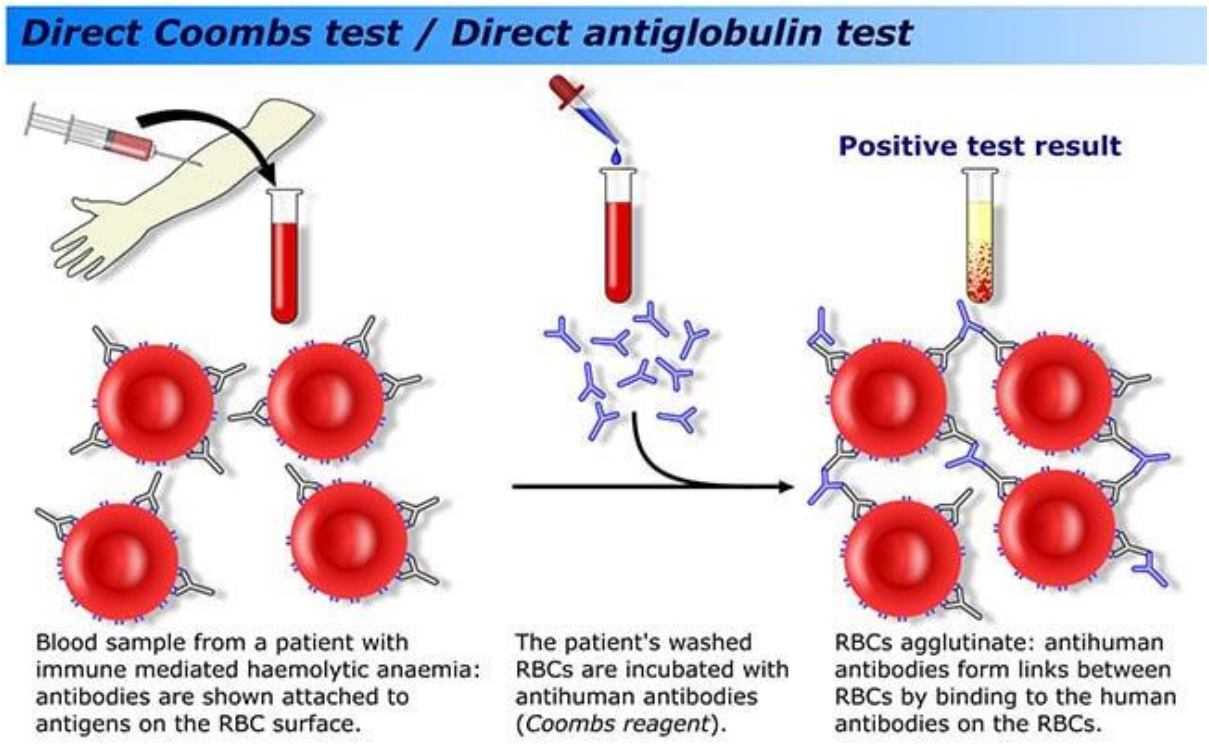
Red cells coated with complement or IgG antibodies do not agglutinate directly when centrifuged. These cells are said to be sensitized with IgG or complement. In order for agglutination to occur an additional antibody, which reacts with the Fc portion of the IgG antibody, or with the C3b or C3d component of complement, must be added to the system. This will form a "bridge" between the antibodies or complement coating the red cells, causing agglutination.

Types of Coombs test

Direct Coombs Test (Direct Antiglobulin Test- DAT)

The direct Coombs test is used to detect antibodies (IgG or C3) that are stuck to the surface of red blood cells. Many diseases and drugs can cause this. These antibodies sometimes destroy red blood cells and cause anemia.

This is the test that is done on the newborn's blood sample, usually in the setting of a newborn with jaundice. The two most commonly recognized forms of antibody-mediated hemolysis in newborns are Rh incompatibility and ABO incompatibility.



Procedure of Direct Coombs Test

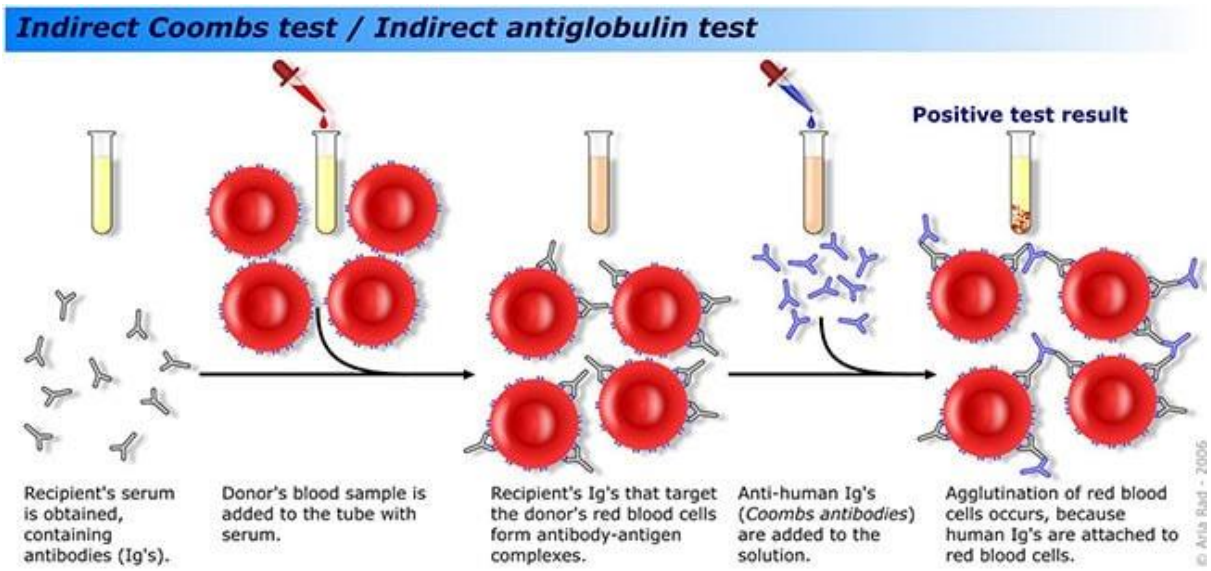
1. Prepare a 5 % suspension in isotonic saline of the red blood cells to be tested.
2. With clean pipette add one drop of the prepared cell suspension to a small tube.
3. Wash three times with normal saline to remove all the traces of serum.
4. Decant completely after the last washing.
5. Add two drops of Anti-human serum.
6. Mix well and centrifuge for one minute at 1500 RPM.
7. Resuspend the cells by gentle agitation and examine macroscopically and microscopically for agglutination.

Indirect Coombs Test (Indirect Antiglobulin Test- IAT)

The indirect Coombs test looks for free-flowing antibodies against certain red blood cells. It is most often done to determine if you may have a reaction to a blood transfusion.

This is the test that is done on the mother's blood sample as part of her prenatal labs. Frequently referred to as the "antibody screen", this test identifies a long list of minor antigens that could either cause problems in the newborns or cause problems in the mother if transfusion is necessary.

Approximately 5% of patients have a positive IAT due to IgG antibodies, IgM antibodies, or both.



Procedure of Indirect Coombs Test

1. Label three test tubes as T (test serum) PC (Positive control) and NC (negative control).
2. In the tube labeled as T (Test), take 2 drops of test serum.
3. In the test tube labeled as PC (Positive control), take 1 drop of anti D serum.
4. In the test tube labeled as NC (Negative control), take 1 drop of normal saline.
5. Add one drop of 5 % saline suspension of the pooled 'O' Rho (D) positive cells in each tube.
6. Incubate all the three tubes for one hour at 37°C.
7. Wash the cells three times in normal saline to remove excess serum with no free antibodies, (in the case of inadequate washings of the red cells, negative results may be obtained).
8. Add two drops of Coombs serum (anti human serum) to each tube.
9. Keep for 5 minutes and then centrifuge at 1,500 RPM for one minute.
10. Resuspend the cells and examine macroscopically as well as microscopically.

Result Interpretation of Coombs Test

Negative Result:

No clumping of cells (no agglutination). This means you have no antibodies to red blood cells.

Positive Result:

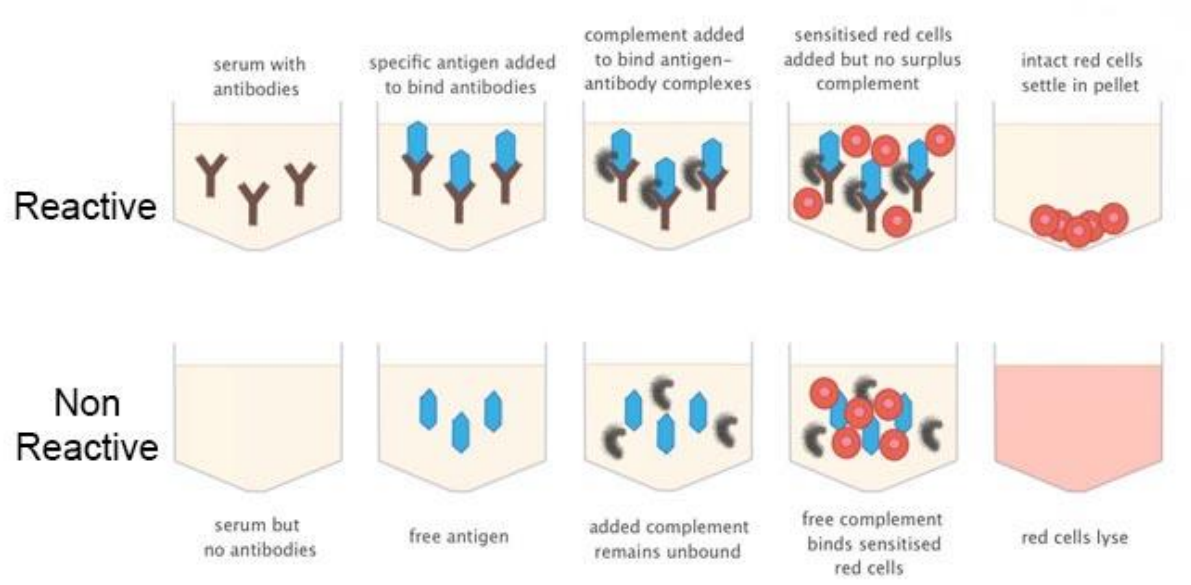
Clumping (agglutination) of the blood cells during a direct Coombs test means that you have antibodies on the red blood cells and that you may have a condition that causes the destruction of red blood cells by your immune system (hemolysis). This may be due to

- Hemolytic anemia,
- Chronic lymphocytic leukemia or similar disorder,
- Erythroblastosis fetalis (hemolytic disease of the newborn),
- Infectious mononucleosis,
- Mycoplasmal infection,
- Syphilis,
- Systemic lupus erythematosus and
- Transfusion reaction, such as one due to improperly matched units of blood.

Complement Fixation Test- Steps, Advantages and Disadvantages

Complement Fixation Test

It is a classic method for demonstrating the presence of antibody in patient serum. It is based on the principle that antigen-antibody complex fixes the complement. As coupling of complement has no visible effects or changes, it is necessary to use an indicator system consisting of sheep RBC and coated with anti-sheep RBC antibody. Complement lyses antibody coated RBC.



The complement fixation test consists of **two components**.

The first component is an **indicator system** that uses combination of sheep red blood cells, complement-fixing antibody such as immunoglobulin G produced against the sheep red blood cells and an exogenous source of complement usually guinea pig serum. When these elements are mixed in optimum conditions, the anti-sheep antibody binds on the surface of red blood cells. Complement subsequently binds to this antigen -antibody complex formed and will cause the red blood cells to lyse.

The second component is **Test System (**A known antigen and patient serum added to a suspension of sheep red blood cells in addition to complement). These two components of the complement fixation method are tested in sequence. Patient serum is first added to the known antigen, and complement is added to the solution. If the serum contains antibody to the antigen, the resulting antigen-antibody complexes will bind all of the complement. Sheep red blood cells and the anti-sheep antibody are then added. If complement has not been bound by an antigen-antibody complex formed from the patient serum and known antigens, it is available to bind to the indicator system of sheep cells and anti-sheep antibody. Lysis of the indicator sheep red blood cells signifies both a lack of antibody in patient serum and a negative complement fixation test. If the patient's serum does contain a complement-fixing antibody, a positive result will be indicated by the lack of red blood cell lysis.

Steps of Complement Fixation Test

Step 1: A known antigen and inactivated patient's serum are incubated with a standardized, limited amount of complement. If the serum contains specific, complement activating antibody the

complement will be activated or fixed by the antigen-antibody complex. However, if there is no antibody in the patient's serum, there will be no formation of antigen-antibody complex, and therefore complement will not be fixed. But will remain free.

Step 2: The second step detects whether complement has been utilized in the first step or not. This is done by adding the indicator system. If the complement is fixed in the first step owing to the presence of antibody there will be no complement left to fix to the indicator system. However, if there is antibody in the patient's serum, there will be no antigen-antibody complex, and therefore, complement will be present free or unfixed in the mixture. This unfixed complement will now react with the antibody- coated sheep red blood cells to bring about their lysis.

Thus, no lysis of sheep red blood cells (positive CFT) indicates the presence of antibody in the test serum, while lysis of sheep red blood cells (Negative CFT) indicates the absence of antibody in the serum.

Advantages of Complement Fixation Test

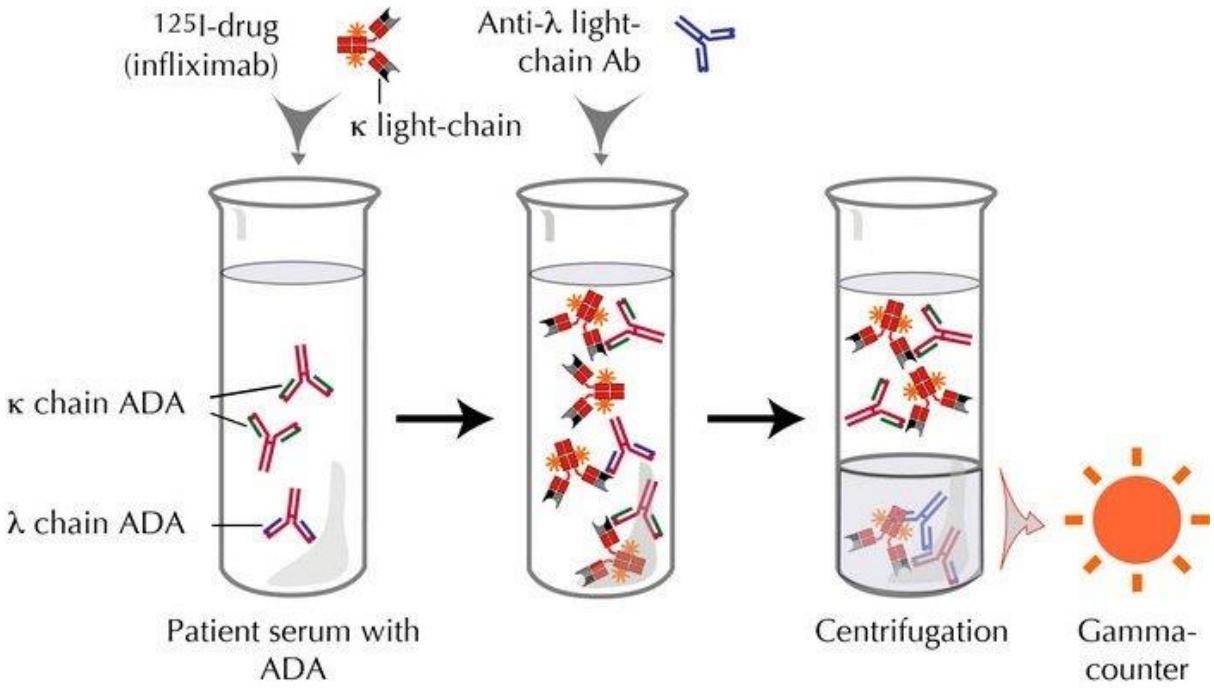
1. Ability to screen against a large number of viral and bacterial infections at the same time.
2. Economical.

Disadvantages of Complement Fixation Test

1. Not sensitive – cannot be used for immunity screening.
2. Time-consuming.
3. Often non-specific e.g. cross-reactivity between Herpes Simplex Virus and Voricella Zoster Virus.

Radioimmunoassay- Principle, Uses and Limitations

When radioisotopes instead of enzymes are used as labels to be conjugated with antigens or antibodies, the technique of detection of the antigen-antibody complex is called radioimmunoassay (RIA). Radioimmunoassay (RIA) is an *in vitro* assay that measures the presence of an antigen with very high sensitivity. RIA was first described in 1960 for the measurement of endogenous plasma insulin by **Solomon Berson and Rosalyn Yalow** of the Veterans Administration Hospital in New York.



The classical RIA methods are based on the principle of competitive binding. In this method, an unlabeled antigen competes with a radiolabeled antigen for binding to an antibody with the appropriate specificity. Thus, when mixtures of radiolabeled and unlabeled antigen are incubated with the corresponding antibody, the amount of free (not bound to antibody) radiolabeled antigen is directly proportional to the quantity of unlabeled antigen in the mixture.

Principle of Radioimmunoassay

It involves a combination of three principles.

1. An immune reaction i.e. antigen, antibody binding.
2. A competitive binding or competitive displacement reaction. (It gives specificity)
3. Measurement of radio emission. (It gives sensitivity)

Immune Reaction:

When a foreign biological substance enters into the body bloodstream through a non-oral route, the body recognizes the specific chemistry on the surface of foreign substance as antigen and produces specific antibodies against the antigen so as nullify the effects and keep the body safe. The antibodies are produced by the body's immune system so, it is an immune reaction. Here the antibodies or antigens bind move due to chemical influence. This is different from principle of electrophoresis where proteins are separated due to charge.

Competitive binding or competitive displacement reaction:

This is a phenomenon wherein when there are two antigens that can bind to the same antibody, the antigen with more concentration binds extensively with the limited antibody displacing others. So here in the experiment, a radiolabelled antigen is allowed to bind to high-affinity antibody. Then when the patient serum is added unlabeled antigens in it start binding to the antibody displacing the labeled antigen.

Measurement of radio emission:

Once the incubation is over, then washings are done to remove any unbound antigens. Then radio emission of the antigen-antibody complex is taken, the gamma rays from radiolabeled antigen are measured.

The target antigen is labeled radioactively and bound to its specific antibodies (a limited and known amount of the specific antibody has to be added). A sample, for e.g. blood-serum, is added in order to initiate a competitive reaction of the labeled antigens from the preparation, and the unlabeled antigens from the serum-sample, with the specific antibodies. The competition for the antibodies will release a certain amount of labeled antigen. This amount is proportional to the ratio of labeled to an unlabeled antigen. A binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived. That means as the concentration of unlabeled antigen is increased, more of it binds to the antibody, displacing the labeled variant. The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigens remaining in the supernatant is measured.

Antigen-antibody complexes are precipitated either by crosslinking with a second antibody or by means of the addition of reagents that promote the precipitation of antigen-antibody complexes. Counting radioactivity in the precipitates allows the determination of the amount of radiolabeled antigen precipitated with the antibody. A standard curve is constructed by plotting the percentage of antibody-bound radiolabeled antigen against known concentrations of a standardized unlabeled antigen, and the concentrations of antigen in patient samples are extrapolated from that curve.

The **extremely high sensitivity** of RIA is its **major advantage**.

Uses of Radioimmunoassay

1. The test can be used to determine very small quantities (e.g. nanogram) of antigens and antibodies in the serum.
2. The test is used for quantitation of hormones, drugs, HBsAg, and other viral antigens.

3. Analyze nanomolar and picomolar concentrations of hormones in biological fluids.

The **limitations of the RIA** include:

1. The cost of equipment and reagents
2. Short shelf-life of radiolabeled compounds
3. The problems associated with the disposal of radioactive waste.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) utilizes an enzyme system to show specific combination of an antigen with its antibody. It is a method of quantifying an antigen immobilized on a solid surface. ELISA uses a specific antibody with a covalently coupled enzyme. The amount of antibody that binds the antigen is proportional to the amount of antigen present, which is determined by spectrophotometrically measuring the conversion of a clear substance to a colored product by the coupled enzyme. The ELISA technique was first conceptualized and developed by **Peter Perlmann** and **Eva Engvall** at Stockholm University, Sweden.

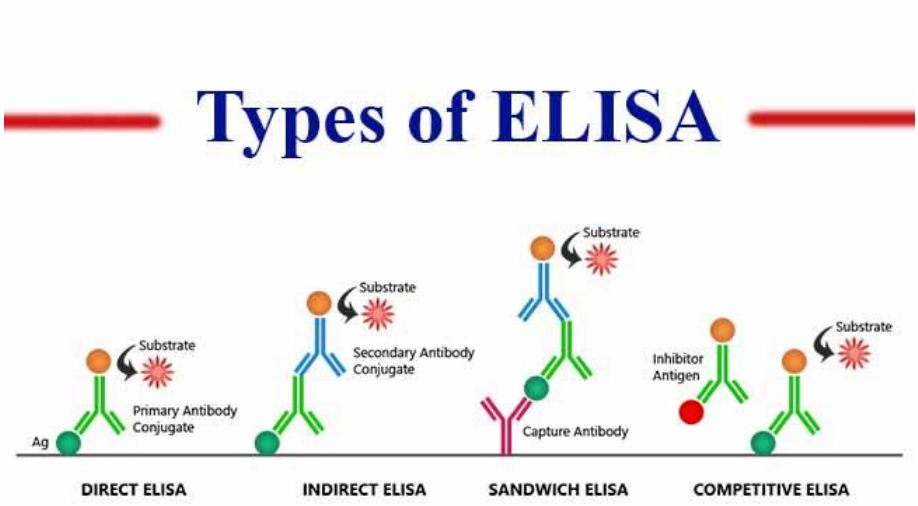
Enzyme system of ELISA consists enzyme which is labeled to a specific antibody or antigen and a chromogenic substrate which is added after antigen-antibody reaction. The substrate is hydrolysed by the enzyme attached to antigen-antibody complexes. An ELISA test uses components of the immune system (such as IgG or IgM antibodies) and chemicals for the detection of immune responses in the body. The ELISA test involves an enzyme (a protein that catalyzes a biochemical reaction). It also involves an antibody or antigen (immunologic molecules). Examples of the uses of an ELISA test includes to diagnose infections such as HIV (human immunodeficiency virus) and some allergic diseases like food allergies. ELISA tests are also known as an immunosorbent assay.

Following the antigen– antibody reaction, chromogenic substrate specific to the enzyme (**o-phenyldiamine dihydrochloride for peroxidase, p-nitrophenyl phosphate for alkaline phosphatase**, etc.) is added. The substrate is acted upon (usually hydrolysed) by enzyme attached to antigen-antibody complex to give color change. The color in reaction can be read visually or The reaction is detected by reading the optical density (estimated colorimetrically) using microassay plate reader i.e. ELISA reader. Usually, a standard curve based on known concentrations of antigen or antibody is prepared from which the unknown quantities are calculated.

The antigen or antibody is coated on solid surface such as in plastic tube or well of microtiter plate. Thus, after the antigen and antibody have combined (Antigen-antibody complex formed) they remain firmly attached to solid surface during subsequent washing stages.

Enzyme immunoassays (EIAs) can be used for detection of either antigens or antibodies in serum and other body fluids of the patient. In EIA techniques, antigen or antibody labeled with enzymes are used. **Alkaline phosphatase, Horseradish peroxidase, and β-galactosidase** are the enzymes used in the EIA tests.

There are different types of ELISAs available for the detection and quantitation of either the antigen or antibodies in serum and other body fluids. There are four types of ELISA tests:



Direct ELISA

Antigen is attached to a polystyrene plate. Enzyme-labeled antibody is added that can react with the antigen and a substrate that can be measured.

Indirect ELISA

Antigen is attached to a polystyrene plate. Addition of primary antibody followed by an enzyme-labeled antibody that can react with both the primary antibody and substrate.

Sandwich ELISA

A capture antibody is attached to the polystyrene plate, then antigen is added that specifically attaches or captures the antigen. A second antibody, also specific for the antigen but not the same as the capture antibody is added and “sandwiches” the antigen. This second antibody is then

followed by an enzyme-labeled antibody specific for the second antibody that can react with a substrate that can be measured.

Immunofluorescence

January 1, 2018 by Sagar Aryal

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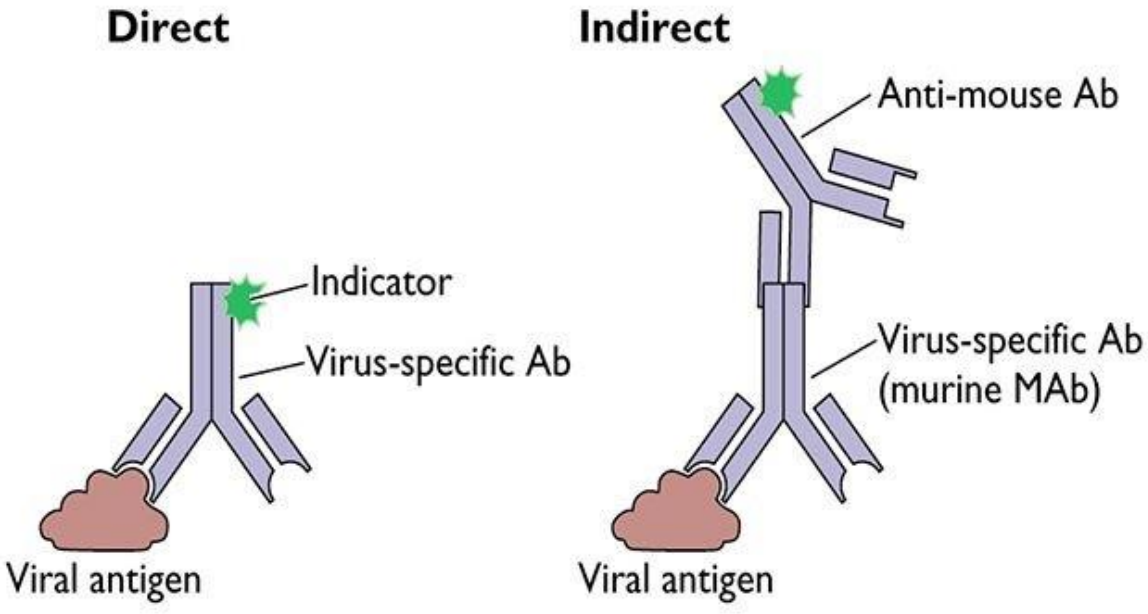
Last Updated on January 9, 2020 by Sagar Aryal

Immunofluorescence is an assay which is used primarily on biological samples and is classically defined as a procedure to detect antigens in cellular contexts using antibodies. The specificity of antibodies to their antigen is the base for immunofluorescence.

The property of certain dyes absorbing light rays at one particular wavelength (ultraviolet light) and emitting them at a different wavelength (visible light) is known as **fluorescence.** In immunofluorescence test, fluorescent dye which illuminates in UV light are used to detect/show the specific combination of an antigen and antibody. The dye usually used is **fluorescein isothiocynate**, which gives yellow-green fluorescence. Immunofluorescence tests are also termed as **fluorescent antibody test (FAT).**



Fluorescent dyes, such as fluorescein isothiocyanate and lissamine rhodamine, can be tagged with antibody molecules. They emit blue-green and orange-red fluorescence, respectively under ultraviolet (UV) rays in the fluorescence microscope. This forms the basis of the immunological test. Immunofluorescence tests have wide applications in research and diagnostics. These tests are broadly of two types:

1. Direct immunofluorescence test
2. Indirect immunofluorescence test

Direct immunofluorescence test

Direct immunofluorescence test is used to detect unknown antigen in a cell or tissue by employing a known labeled antibody that interacts directly with unknown antigen. If antigen is present, it reacts with labeled antibody and the antibody coated antigen is observed under UV light of the fluorescence. It involves use of labeled antiviral antibody.

Method

The specimen is placed on slide; fluorescent labeled antibody is then added to it and allowed for some time for Antigen-Antibody reaction. The preparation is then washed which will allow the removal of other components except the complex of antigen and fluorescent labeled antibody. On microscopy (Fluorescence Microscopy), Antigen- Antibody complex are observed fluorescing due to the dye attached to antibody.

The need for preparation of separate labeled antibody for each pathogen is the major disadvantage of the direct immunofluorescence test.

Indirect immunofluorescence test

Indirect fluorescence is a double antibody technique. The unlabeled antibodies which have bound to the antigens are visualized by a fluorescent antiglobulin reagent directed at the unlabeled antibodies. The indirect immunofluorescence test is used for detection of specific antibodies in the serum and other body fluids for sero-diagnosis of many infectious diseases.

Method

Indirect immunofluorescence is a two-stage process.

First stage: A known antigen is fixed on a slide. Then the patient's serum to be tested is applied to the slide, followed by careful washing. If the patient's serum contains antibody against the antigen, it will combine with antigen on the slide.

Second stage: The combination of antibody with antigen can be detected by addition of a fluorescent dye-labeled antibody to human IgG, which is examined by a fluorescence microscope.

The first step in the indirect immunofluorescence test is the incubation of a fixed antigen (e.g., in a cell or tissue) with unlabeled antibody, which becomes associated with the antigen. After careful washing, a fluorescent antibody (e.g. fluorescent labeled anti-IgG) is added to the smear. This second

antibody will become associated to the first, and the antigen–antibody complex can be visualized on the fluorescence microscope.

The indirect method has the advantage of using a single labeled antiglobulin (antibody to IgG) as a "universal reagent" to detect many different specific antigen–antibody reactions. The test is often more sensitive than the direct immunofluorescence test.

Indirect immunofluorescence test is used widely to:

1. Detect specific antibodies for serodiagnosis of syphilis, leptospirosis, amoebiasis, toxoplasmosis, and many other infectious diseases;
2. Identify the class of a given antibody by using fluorescent antibodies specific for different immunoglobulin isotypes;
3. Identify and enumerate lymphocyte subpopulations by employing monoclonal antibodies and cytofluorographs; and
4. Detect autoantibodies, such as antinuclear antibodies in autoimmune diseases.

Immunofluorescence may also be used to analyze the distribution of proteins, glycans, and small biological and non-biological molecules. Immunofluorescence has been widely used in biological research and medical research.

The major limitation of immunofluorescence is that the technique requires

1. expensive fluorescence microscope and reagents,
2. trained personnel
3. have a factor of subjectivity that may result in erroneous results

The biological samples include tissue and cells. Immunofluorescence aid to evaluate whether or not cells in a particular sample express the antigen in study. In cases where an immune-positive signal is found, immunofluorescence also helps to determine which subcellular compartments are expressing the antigen. Immunofluorescence can be used on cultured cell lines, tissue sections, or individual cells.

Western blotting- Introduction, Principle and Applications

October 23, 2017 by Sagar Aryal

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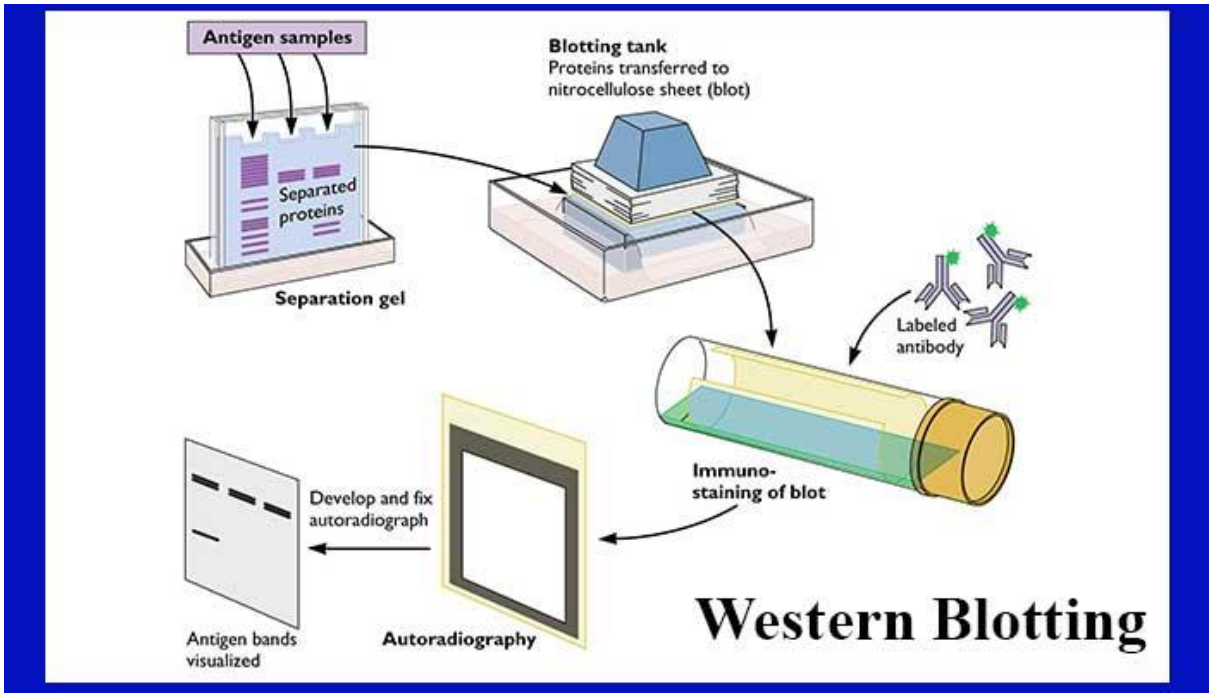
Last Updated on January 6, 2020 by Sagar Aryal

Introduction

Western blot is the analytical technique used in molecular biology, immunogenetics and other molecular biology to detect specific proteins in a sample of tissue homogenate or extract. Western blotting is called so as the procedure is similar to Southern blotting. While Southern blotting is done to detect DNA, Western blotting is done for the detection of proteins. Western blotting is also called protein immunoblotting because an antibody is used to specifically detect its antigen.



Principle of Western blotting

The technique consists of three major processes:

1. Separation of proteins by size (Electrophoresis).
2. Transfer to a solid support (Blotting)
3. Marking target protein using a proper primary and secondary antibody to visualize (Detection).

Electrophoresis used to separate proteins according to their electrophoretic mobility which depends on charge, size of protein molecule and structure of the proteins. Proteins are moved from within the gel onto a membrane made of Nitrocellulose (NC) or Polyvinylidene difluoride (PVDF). Without pre-activation, proteins combine with nitrocellulose membrane based on hydrophobic interaction (**Blotting**). For detection of the proteins primary antibody and enzyme conjugated secondary antibody are used. On addition of substrate, a substrate reacts with the enzyme that is bound to the secondary antibody to generate colored substance, namely, visible protein bands.

In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present; thus doing a standard can indicate the amount of protein present.

Western blotting is usually done on a tissue homogenate or extract. It utilizes **SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis)**, a type of gel electrophoresis to first separate various proteins in a mixture on the basis of their shape and size. The protein bands thus obtained are transferred onto a **nitrocellulose or nylon membrane** where they are "probed" with antibodies specific to the protein to be detected. The antigen–antibody complexes that form on the band containing the protein recognized by the antibody can be visualized in a variety of ways.

If the protein of interest is bound by a radioactive antibody, its position on the blot can be determined by exposing the membrane to a sheet of X-ray film, a procedure called **autoradiography**. However, the most generally used detection procedures employ enzyme-linked antibodies against the protein. After binding of the enzyme–antibody conjugate, addition of a chromogenic substrate that produces a highly colored and insoluble product causes the appearance of a colored band at the site of the target antigen. The site of the protein of interest can be determined with much higher sensitivity if a chemiluminescent compound along with suitable enhancing agents is used to produce light at the antigen site.

Applications of Western blotting

1. Identification of a specific protein in a complex mixture of proteins. In this method, known antigens of well-defined molecular weight are separated by SDS-PAGE and blotted onto nitrocellulose. The separated bands of known antigens are then probed with the sample suspected of containing antibody specific for one or more of these antigens. Reaction of an antibody with a band is detected by using either radiolabeled or enzyme-linked secondary antibody that is specific for the species of the antibodies in the test sample.
2. Estimation of the size of the protein as well as the amount of protein present in the mixture.

3. It is most widely used as a confirmatory test for diagnosis of HIV, where this procedure is used to determine whether the patient has antibodies that react with one or more viral proteins or not.
4. Demonstration of specific antibodies in the serum for diagnosis of neurocysticercosis and tubercular meningitis.

Endotoxin Testing (LAL)

Endotoxin testing (LAL test) ensures injectable therapeutics are safe for human use.

Bacterial endotoxins are lipopolysaccharides (LPS), components of Gram-negative bacterial cell walls known to cause fevers and disease when injected into the bloodstream. Bacterial endotoxins are heat stable and toxicity is not dependent on the presence of the bacterial cell.

Since many therapeutics are made in bacteria, **endotoxin testing** is employed to ensure a therapeutic product is endotoxin-free. BioReliance tests for endotoxin in several areas of drug manufacturing and production including:

- Bulk Lot Release Testing
- Final Product Release Testing
- Raw Materials Testing

BioReliance research professionals have performed thousands of endotoxin testing assays for clients in Biopharma. We can provide a fast turnaround time and the highest quality testing to produce therapeutics that are bacterial endotoxin-free and safe for use in humans.

To see our endotoxin testing assays, use the search tool above.

How is endotoxin testing performed (the LAL test)?

The most common approach to endotoxin testing is the limulous amoebocyte lysate test (**LAL test**). This assay is based in the biology of the horseshoe crab (*Limulous*). These animals produce LAL enzymes in blood cells (amoebocytes) to bind and inactivate endotoxin from invading bacteria.

LAL serves as a primitive immune system. Inactivation of endotoxin also forms a clot, which can further protect the horseshoe crab from infection. The LAL test exploits the action of this enzyme, by adding LAL reagent to the tested product, and assaying for clot formation.

BioReliance offers LAL - endotoxin tests with a variety of assay options including:

- Quantitative and qualitative testing
- Gel-clot method (LAL test)
- Chromogenic methods (USP Chromogenic)

The LAL test was accepted by the FDA for endotoxin testing of pharmaceuticals and medical devices in 1983. USP requires pooled testing of a production lot for the presence of bacterial endotoxin.

To find the right endotoxin testing assay, use the search tool to the right or click here.

Endotoxin testing - the gel clot method vs. the chromogenic assay.

Both the gel clot method and the chromogenic method of endotoxin testing are appropriate for preclinical, Phase 1, Phase 2, Phase 3, and Licensed Product phases of therapeutic production and distribution.

Either test is approved for endotoxin testing of multiple therapeutic types including cell therapy, recombinant protein, monoclonal antibody, vaccines and gene therapy. The choice comes down to whether you need quantitative results or simply to detect endotoxin in your drug.

The gel clot assay with the LAL test is simplest method for the detection of bacterial endotoxins and the GMP format is typically used for lot release of final product intended for injection in humans.

To perform the assay, equal volumes of LAL reagents are mixed with undiluted or diluted test article and observed for clot formation. The dilutions are selected to cover the potential range of endotoxin in the sample and to reduce interference by the test material making the gel clot LAL test semi-quantitative. The levels of endotoxin allowed are strictly regulated and depend on the route and frequency of administration. The sensitivity of this assay is 0.06 EU/ml.

The USP chromogenic method is based on the activation of a serine protease (coagulase) by the endotoxin, which is the rate-limiting step of the clotting cascade. The assay measures the activation of the serine protease as opposed to the end result of this activation, which is clotting. The natural substrate, coagulogen, is replaced by a chromogenic substrate. On cleavage of this substrate a chromophore is released from the chromogenic peptide and is measured by spectrophotometry.

The USP chromogenic method is quantitative and can provide a greater sensitivity over a wider range. The sensitivity of this assay is 0.10 EU/ml.

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SCHOOL OF BIO AND CHEMICAL ENGINEERNG

DEPARTMENT OF BIOTECHNOLOGY

UNIT – II – Humoral Immunity – SBTA1402

I. HUMORAL IMMUNITY

Antigen- Properties, Types and Determinants of Antigenicity

Last updated: September 26, 2018 by Sagar Aryal

Antigen is a substances usually protein in nature and sometimes polysaccharide, that generates a specific immune response and induces the formation of a specific antibody or specially sensitized T cells or both.

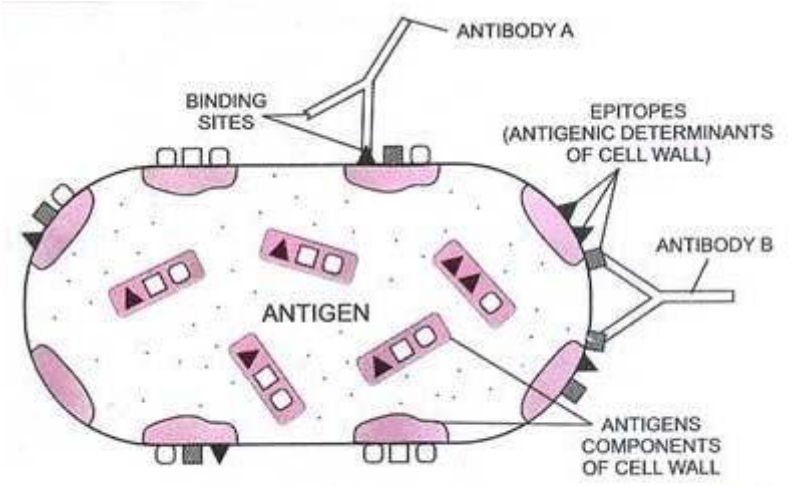


Diagram showing an antigen with epitopes (antigenic determinants). Two attached antibodies are also shown.\

Although all antigens are recognized by specific lymphocytes or by antibodies, only some antigens are capable of activating lymphocytes. Molecules that stimulate immune responses are called I**mmunogens.**

Epitope is immunologically active regions of an immunogen (or antigen) that binds to antigen-specific membrane receptors on lymphocytes or to secreted antibodies. It is also called **antigenic determinants.**

Autoantigens, for example, are a person's own self antigens. Examples: Thyroglobulin, DNA, Corneal tissue, etc.

Alloantigens are antigens found in different members of the same species (the red blood cell antigens A and B are examples).

Heterophile antigens are identical antigens found in the cells of different species. Examples: Forrssman antigen, Cross-reacting microbial antigens, etc.

Adjuvants are substances that are non-immunogenic alone but enhance the immunogenicity of any added immunogen.

Chemical Nature of Antigens (Immunogens)

A. Proteins

The vast majority of immunogens are proteins. These may be pure proteins or they may be glycoproteins or lipoproteins. In general, proteins are usually very good immunogens.

B. Polysaccharides

Pure polysaccharides and lipopolysaccharides are good immunogens.

C. Nucleic Acids

Nucleic acids are usually poorly immunogenic. However, they may become immunogenic when single stranded or when complexed with proteins.

D. Lipids

In general lipids are non-immunogenic, although they may be haptens.

Types of Antigen On the basis of order of their class (Origin)

1. Exogenous antigens

- These antigens enters the body or system and start circulating in the body fluids and trapped by the APCs (Antigen processing cells such as macrophages, dendritic cells, etc.)
- The uptakes of these exogenous antigens by APCs are mainly mediated by the phagocytosis
- Examples: bacteria, viruses, fungi etc
- Some antigens start out as exogenontigens, and later become endogenous (for example, intracellular viruses)

2. Endogenous antigens

- These are body's own cells or sub fragments or compounds or the antigenic products that are produced.
- The endogenous antigens are processed by the macrophages which are later accepted by the cytotoxic T – cells.
- Endogenous antigens include xenogenic (heterologous), autologous and idiotypic or allogenic (homologous) antigens.
- Examples: Blood group antigens, HLA (Histocompatibility Leukocyte antigens), etc.

3. Autoantigens

- An autoantigen is usually a normal protein or complex of proteins (and sometimes DNA or RNA) that is recognized by the immune system of patients suffering from a specific autoimmune disease

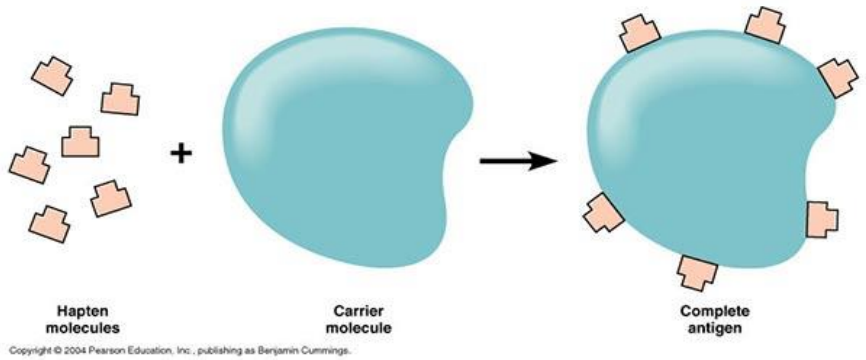
- These antigens should not be, under normal conditions, the target of the immune system, but, due mainly to genetic and environmental factors, the normal immunological tolerance for such an antigen has been lost in these patients.
- Examples: Nucleoproteins, Nucleic acids, etc.

On the basis of immune response

1. Complete Antigen or Immunogen

- Posses antigenic properties denovo, i.e. ther are able to generate an immune response by themselves.
- High molecular weight (more than 10,000)
- May be proteins or polysaccharides

2. Incomplete Antigen or Hapten



- These are the foreign substance, usually non-protein substances
- Unable to induce an immune response by itself, they require carrier molecule to act as a complete antigen.
- The carrier molecule is a non-antigenic component and helps in provoking the immune response. Example: Serum Protein such as Albumin or Globulin.
- Low Molecular Weight (Less than 10,000)
- Haptens can react specifically with its corresponding antibody.
- Examples: Capsular polysaccharide of pneumococcus, polysaccharide "C" of beta haemolytic streptococci, cardiolipin antigens, etc.

Determinants of Antigenicity

The whole antigen does not evoke immune response and only a small part of it induces B and T cell response.

The small area of chemical grouping on the antigen molecule that determines specific immune response and reacts specifically with antibody is called an ***antigenic determinant.***

Property of antigens/ Factors Influencing Immunogenicity

Immunogenicity is determined by:

1. Foreignness

- An antigen must be a foreign substances to the animal to elicit an immune response.

2. Molecular Size

- The most active immunogens tend to have a molecular mass of 14,000 to 6,00,000 Da.
- Examples: tetanus toxoid, egg albumin, thyroglobulin are highly antigenic.
- Insulin (5700) are either non-antigenic or weakly antigenic.

3. Chemical Nature and Composition

- In general, the more complex the substance is chemically the more immunogenic it will be.
- Antigens are mainly proteins and some are polysaccharides.
- It is presumed that presence of an aromatic radical is essential for rigidity and antigenicity of a substance.

4. Physical Form

- In general particulate antigens are more immunogenic than soluble ones.
- Denatured antigens are more immunogenic than the native form.

5. Antigen Specificity

- Antigen Specificity depends on the specific actives sites on the antigenic molecules (Antigenic determinants).
- Antigenic determinants or epitopes are the regions of antigen which specifically binds with the antibody molecule.

6. Species Specificity

- Tissues of all individuals in a particular species possess, species specific antigen.
- Human Blood proteins can be differentiated from animal protein by specific antigen-antibody reaction.

7. Organ Specificity

- Organ specific antigens are confined to particular organ or tissue.
- Certain proteins of brain, kidney, thyroglobulin and lens protein of one species share specificity with that of another species.

8. Auto-specificity

- The autologous or self antigens are ordinarily not immunogenic, but under certain circumstances lens protein, thyroglobulin and others may act as ***autoantigens.***

9. Genetic Factors

- Some substances are immunogenic in one species but not in another .Similarly, some substances are immunogenic in one individual but not in others (i.e. responders and non-responders).
- The species or individuals may lack or have altered genes that code for the receptors for antigen on B cells and T cells.
- They may not have the appropriate genes needed for the APC to present antigen to the helper T cells.

10. Age

- Age can also influence immunogenicity.
- Usually the very young and the very old have a diminished ability to elicit and immune response in response to an immunogen.

11. Degradability

- Antigens that are easily phagocytosed are generally more immunogenic.
- This is because for most antigens (T-dependant antigens) the development of an immune response requires that the antigen be phagocytosed, processed and presented to helper T cells by an antigen presenting cell (APC).

12. Dose of the antigen

- The dose of administration of an immunogen can influence its immunogenicity.
- There is a dose of antigen above or below which the immune response will not be optimal.

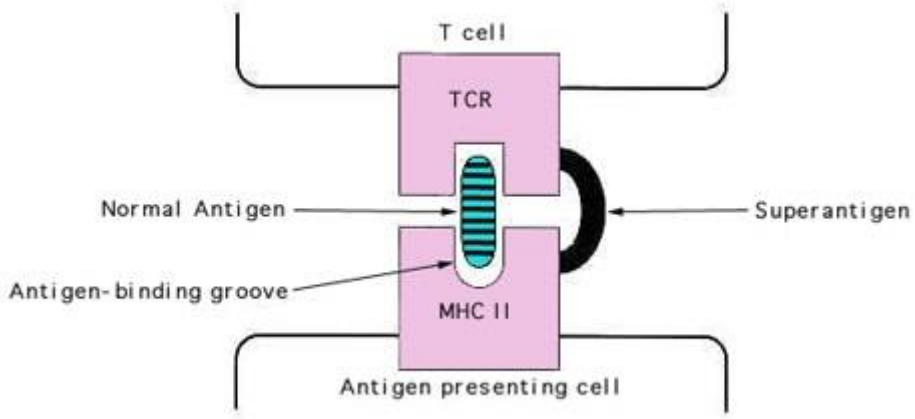
13. Route of Administration

- Generally the subcutaneous route is better than the intravenous or intragastric routes.
- The route of antigen administration can also alter the nature of the response.
- Antigen administered intravenously is carried first to the spleen, whereas antigen administered subcutaneously moves first to local lymph nodes.

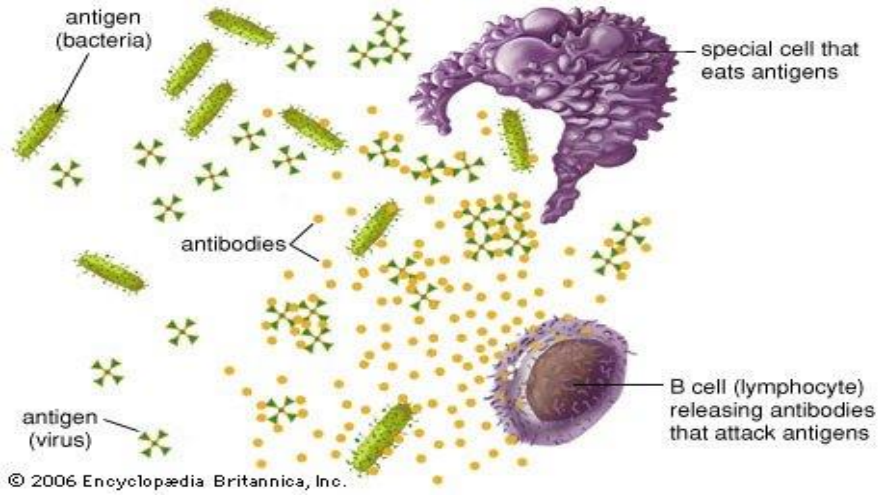
14. Adjuvants

- Substances that can enhance the immune response to an immunogen are called adjuvants.
- The use of adjuvants, however, is often hampered by undesirable side effects such as fever and inflammation.
- Example: aluminum hydroxide.

Superantigens

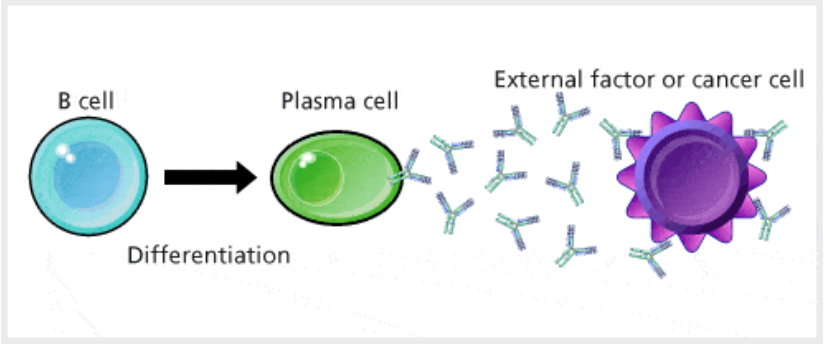


- When the immune system encounters a conventional T-dependent antigen, only a small fraction (1 in 104 -105) of the T cell population is able to recognize the antigen and become activated (monoclonal/oligoclonal response).
- However, there are some antigens which polyclonally activate a large fraction of the T cells (up to 25%). These antigens are called superantigens.
- Examples of superantigens include: Staphylococcal enterotoxins (food poisoning), Staphylococcal toxic shock toxin (toxic shock syndrome), Staphylococcal exfoliating toxins (scalded skin syndrome) and Streptococcal pyrogenic exotoxins (shock).
- Although the bacterial superantigens are the best studied there are superantigens associated with viruses and other microorganisms as well.
- The diseases associated with exposure to superantigens are, in part, due to hyper activation of the immune system and subsequent release of biologically active cytokines by activated T cells.



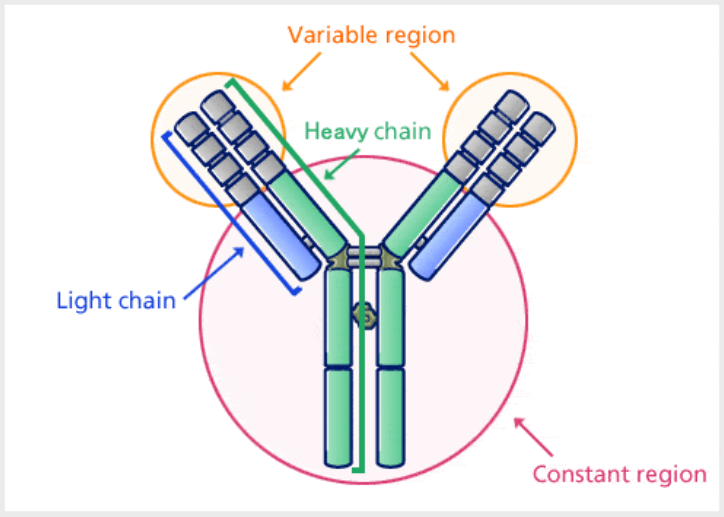
Antibody- Structure, Classes and Functions

Antibody (Ab) also know as Immunoglobulin (Ig) is the large Y shaped protein produced by the body's immune system when it detects harmful substances, called antigens like bacteria and viruses. The production of antibodies is a major function of the immune system and is carried out by a type of white blood cell called a B cell (B lymphocyte), differentiated B cells called plasma cells. The produced antibodies bind to specific antigens express in external factors and cancer cells.



Structure of Antibody

Antibodies are heavy (~150 kDa) globular plasma proteins. The basic structure of all antibodies are same.

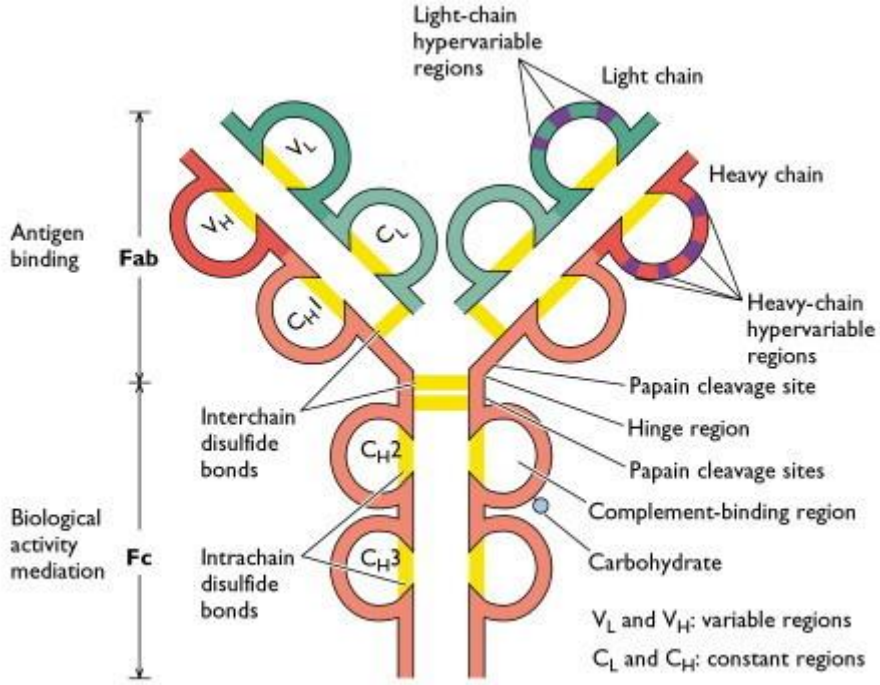


Structure of Antibody.

There are four polypeptide chains: two identical **heavy chains** and two identical **light chains** connected by disulfide bonds. Light Chain (L) consists polypeptides of about 22,000 Da and Heavy Chain (H) consists larger polypeptides of around 50,000 Da or more. There are five

types of Ig **heavy chain** (in mammal) denoted by the Greek letters: α, δ, ε, γ, and μ. There are two types of Ig **light chain** (in mammal), which are called lambda (λ) and kappa (κ).

An antibody is made up of a variable region and a constant region, and the region that changes to various structures depending on differences in antigens is called the **variable region**, and the region that has a constant structure is called the **constant region.**

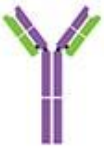
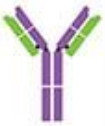


Structure of Antibody

Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region that consists of 100 to 110 amino acids and differ from one antibody to another. The remainder of each chain in the molecule – the constant (C) region exhibits limited variation that defines the two light chain subtypes and the five heavy chains subclasses. Some heavy chains (α, δ, γ) also contain a proline-rich hinge region. The amino terminal portions, corresponding to the V regions, bind to antigen; effector functions are mediated by the carboxy-terminal domains. The ε and μ heavy chains, which lack a hinge region, contain an additional domain in the middle of the molecule. CHO denotes a carbohydrate group linked to the heavy chain.

Classes/Types of Antibody

Serum containing antigen-specific antibodies is called antiserum. The 5 types – IgG, IgM, IgA, IgD, IgE – (isotypes) are classified according to the type of heavy chain constant region, and are distributed and function differently in the body.

The Five Immunoglobulin (Ig) Classes					
	IgM pentamer	IgG monomer	Secretory IgA dimer	IgE monomer	IgD monomer
					
Heavy chains	μ	γ	α	ε	δ
Number of antigen binding sites	10	2	4	2	2
Molecular weight (Daltons)	900,000	150,000	385,000	200,000	180,000
Percentage of total antibody in serum	6%	80%	13%	0.002%	1%
Crosses placenta	no	yes	no	no	no
Fixes complement	yes	yes	no	no	no
Fc binds to		phagocytes		mast cells and basophils	
Function	Main antibody of primary responses, best at fixing complement; the monomer form of IgM serves as the B cell receptor	Main blood antibody of secondary responses, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva, colostrum	Antibody of allergy and antiparasitic activity	B cell receptor

Functions of Antibody

1. IgG provides long term protection because it persists for months and years after the prescence of the antigen that has triggered their production.
2. IgG protect against bacteris, viruses, neutralise bacterial toxins, trigger compliment protein systems and bind antigens to enhance the effectiveness of phagocytosis.
3. Main function of IgA is to bind antigens on microbes before they invade tissues. It aggregates the antigens and keeps them in the secretions so when the secretion is expelled, so is the antigen.
4. IgA are also first defense for mucosal surfaces such as the intestines, nose, and lungs.
5. IgM is involved in the ABO blood group antigens on the surface of RBCs.
6. IgM enhance ingestions of cells by phagocytosis.
7. IgE bind to mast cells and basophils wich participate in the immune response.
8. Some scientists think that IgE’s purpose is to stop parasites.

9. IgD is present on the surface of B cells and plays a role in the induction of antibody production.

There are five immunoglobulin classes of antibody molecules found in serum: IgG, IgM, IgA, IgE and IgD. They are distinguished by the type of heavy chain they contain.

- **IgG antibody structure and function**

Immunoglobulin G (IgG) antibodies are large globular proteins with a molecular weight of about 150 kDa made of four peptide chains. It contains two identical γ (gamma) heavy chains of about 50 kDa and two identical light chains of about 25 kDa, thus a tetrameric quaternary structure.

IgG provides long term protection because it persists for months and years after the prescence of the antigen that has triggered their production. IgG protect against bacteria, viruses, neutralise bacterial toxins, trigger compliment protein systems and bind antigens to enhance the effectiveness of phagocytosis.

- **IgM antibody structure and function**

Immunoglobulin M (IgM) antibodies are constructed of five or six units (i.e. mostly as pentamers but also hexamers occur) which are each comprised of two heavy-chains (μ-chains) and two light chains, bound together by disulfide bonds and a so-called J-chain.

IgM is involved in the ABO blood group antigens on the surface of RBCs. IgM enhance ingestions of cells by phagocytosis.

- **IgA antibody structure and function**

Immunoglobulin A (IgA) antibodies consist of heavy (H) and light (L) chains. Each H chain is comprised of the constant region (Cα1, Cα2, Cα3), hinge region and the Variable (V) region. Light chains consists of the CL and Vκ or Vλ elements.

Main function of IgA is to bind antigens on microbes before they invade tissues. It aggregates the antigens and keeps them in the secretions so when the secretion is expelled, so is the antigen. IgA are also first defense for mucosal surfaces such as the intestines, nose, and lungs.

- **IgE antibody structure and function**

Immunoglobulin E (IgE) antibodies have only been found in mammals. IgE is synthesised by plasma cells. Monomers of IgE consist of two heavy chains (ε chain) and two light chains, with the ε chain containing 4 Ig-like constant domains (Cε1-Cε4).

IgE bind to mast cells and basophils which participate in the immune response. Some scientists think that IgE's purpose is to stop parasites.

- **IgD antibody structure and function**

Immunoglobulin D (IgD) antibodies are expressed in the plasma membranes of immature B-lymphocytes. IgD is also produced in a secreted form that is found in small amounts in blood serum. IgD plays a role in the induction of antibody production.

Antibody formats:

Chimeric antibodies can be generated by fairly straightforward genetic engineering, by joining the immunoglobulin (Ig) variable regions of a selected mouse hybridoma to human Ig constant regions, and be used as such or as a first stage towards further humanization.

Anti-idotypic antibody is an antibody that binds to the idiotype of another antibody. An Idiotype (ID) actually consists of multiple antigenic determinants, each of which is an idiotope. The antigenic determinants or idiotopes can reside in the heavy chain component of the V region, in its light chain component, or they may consist of a surface made up of parts of both chains.

The bispecific antibody can override the specificity of an effector cell for its natural target and redirect it to kill a target that it would otherwise ignore. Different cytotoxic cells express different triggering molecules (receptors). Thus, by varying the specificities of target and effector binding domains a variety of effector responses can be directed against most types of target cells. Alternatively, the full range of effector functions (i.e. ADCC, phagocytosis, complement activation and extended serum half-life) can be conferred by targeting one binding specificity to serum immunoglobulin.

Recombinant antibodies are monoclonal antibodies produced by recombinant DNA technology. Owing to their high specificity, sensitivity and reproducibility, recombinant antibodies are widely used in biomedical science and medicine.

Antigenic Determinants on Immunoglobulins:

Since antibodies are glycoproteins,they can themselves function as potent immunogens to induce an antibody response.Such anti-Ig antibodies are powerful tools for the study of B-cell development and humoral immune responses.The antigenic determinants,or epitopes,on immunoglobulin molecules fall into three major categories:isotypic,allotypic,and idiotypic determinants,which are located in characteristic portions of the molecule.

Isotypic Determinants:

Isotype determinants are consonant-region determinants that collectively define each heavy-chain class and sub class and each light-chain type and subtype within a species.Each isotype is encoded by a separate consonant region gene,and all members of a species carry the same consonant-region genes(which may include multiple alleles).Within a species,each normal

individual will express all isotypes.Therefore,when an antibody from one species,each normal individual will express all isotypes in the serum.Different species inherit different constant-region genes and therefore express different isotypes.Therefore,when an antibody from one species is injected into another species,the isotypic determinants will be recognized as foriegn,inducing an antibody response to the isotypic determinants on the foriegn antibody.Anti-isotype antibody is routinely used for research purposes to determine the class or subclass of serum antibody produced during an immune response or to characterize the class of membrane-bound antibody present on B cells.

Allotypic Determinants:

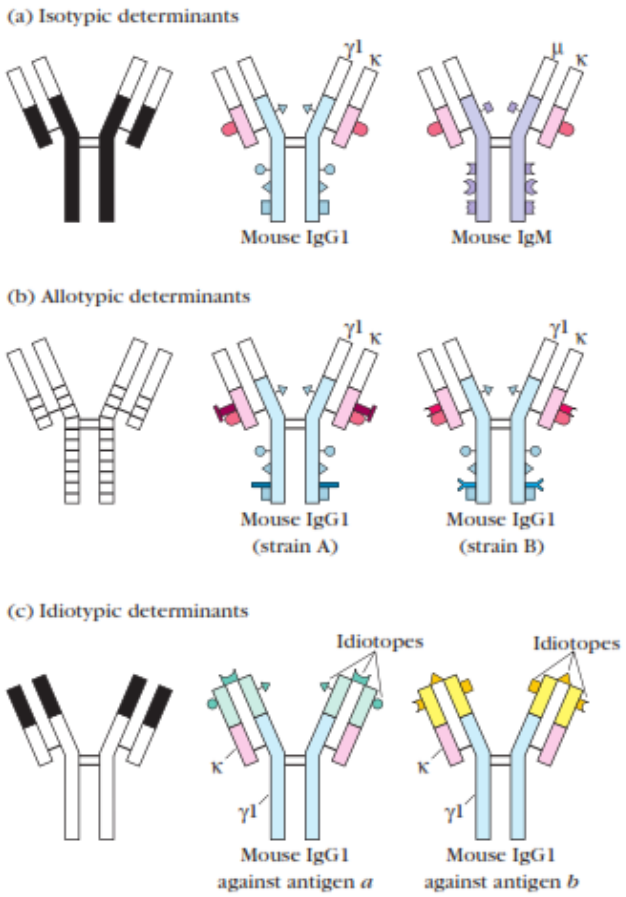
Although all members of a species inherit the same set of isotype genes,multiple alleles exist for some of the genes.These alleles encode subtle amino acid differences,called allotypic determinants displayed by an antibody determines its allotypes.In humans,allotypes have been characterized for all IgG subclasses,for one IgA subclass,and for the light chain.The gamma chain allotypes are referred to as Gm markers.At least 25 different Gm allotypes have been identified;they are designated by the class and subclass followed by the allele number,for example,G1m(1),G2m(23),G3m(11),G4m(4a). Of the two IgA subclasses,only the IgA2 subclass has allotypes,as A2m(1) andA2m(2). The light chain has three allotypes.Each of these allotypic determinants represents differences in one to four amino acids that are encoded by different alleles.

Idiotypic Determinants:

The unique amino acid sequence of the Heavy and light domains of a given antibody can function not only as an antigen-binding site but also as a set of antigenic determinants.The idiotypic determinants arise from the sequence of the heavy and light chain variable regions.Each individual antigenic determinant of the variable region is referred to as an idiotope.In some cases an idiotope maybe the actual antigen-binding site,and in some cases an idiotope may comprise variable region sequences outside of the antigen-binding site.Each antibody will present multiple idiotopes;the sum of the individual idiotopes is called idiotype of the antibody.

Because the antibodies produced by individual B cells derived from the clone have identical variable-region sequences,they all have the same idiotype.Anti-idiotype antibody is produced by injecting antibodies that have minimal variation in their isotypes and allotypes ,so that the idiotypic differences can be recognized.Often a homogenous antibody such as myeloma protein or monoclonal antibody is used.Injection of such an antibody into a recipient who is genetically

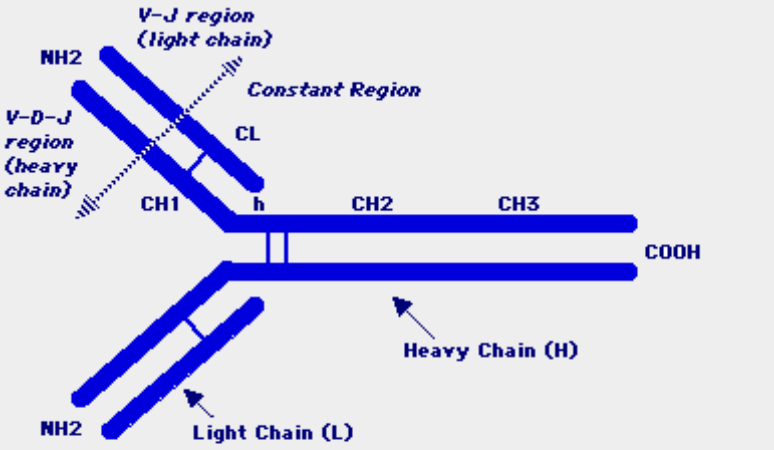
identical to the donor will result in formation of anti-idiotype antibody to the idiotypic determinants.



Immunoglobulin Genes

Introduction

An immunoglobulin (Ig) consists of 2 identical light chains (L) and 2 identical heavy chains (H) (for example IgG-type); at the three-dimensional level, an Ig chain consists of one N-terminal variable domain, V, and one (for an L chain) or several (for an H chain) C-terminal constant domain(s), C.The cells of the B line synthesize immunoglobulins. They are either produced at a membrane (on the surface of the B-lymphocytes) or are secreted (by the plasmocytes).



The variable V-J region of the light chain and the variable V-D-J region of the heavy chain correspond respectively to A variable domain.
The constant region CL of the light chain corresponds to one constant domain.
The constant region of the heavy chain comprises 4 constant domains CH1 to CH4 (mu and epsilon chain) or 3 constant domains, the CH1 and CH2 domains being separated by the hinge (h) (delta, gamma, and alpha chain).

I. Historical questions

As soon as the main characteristics of the immunoglobulins were discovered, a number of questions arose:

A· The antigens are highly varied; to be able to respond to them, the immunoglobulins must be equally diverse (there are 10^{11} to 10^{12} different Igs!), which corresponds to the diversity of the amino acids of the N-terminal parts of the L and H chains (i.e. to the variable domains).

· Does this reflect extreme diversity of the genes responsible for coding the immunoglobulins? (in line with the model of the germline theory: 1 gene = 1 Ig chain; in which case many genes would have to be implicated; they may arise from the duplication of ancestral genes; but the entire human genome would not suffice to encode all the immunoglobulins!).

· Does this reflect an accumulation of mutations? (in line with the model of the theory of somatic mutations: in this case, only a few genes would be implicated, but numerous somatic mutations would then have to take place to produce the diversity of the immunoglobulins produced; however this model would run counter to the generally accepted principles of genetics).

· Does this reflect a mechanism specific to the immunoglobulin genes?

B · During its differentiation, a B cell, first produces membrane immunoglobulins on the surface of the B-lymphocyte, and then produces the immunoglobulins secreted by the plasmocyte. The amino acid sequence of the heavy chains of the membrane and secreted Igs differ only at their C-terminal end: are the same genes implicated in both cases?

C · A B-cell first expresses the IgM at its surface and then, during its differentiation, may express another class of Ig (IgG, IgE or IgA) (this mechanism is known as an isotype switch): how does this switch occur? How can we explain that regardless of the immunoglobulin isotype produced, the same specific antigen variable domain (same idiotype) is expressed?

D · A B-cell synthesizes a single type of heavy chain and light chain, even though its genome has 2 chromosomes (2 alleles) for each Ig locus; allele exclusion must therefore occur and a hemizygote phenotype is produced; how does this allele exclusion take place?

E · Finally, if the variable regions do undergo mutations, why aren't there any in the constant regions?

Various methods used in molecular biology and gene cloning in the mouse and in human beings have been used to answer these questions; we will limit our discussion to human immunoglobulins.

II. Answers

II.1. Light chains (kappa or lambda)

II.1.1. Kappa chain: V-J rearrangements

II.1.2. Lambda chain: V-J rearrangements

Immunoglobulin Genes

- The organisation of Ig Gene – Highly complex – each Ab against limitless array of foreign Ag – displays unique AA sequence in V region – limited No. of invariant sequence in C region
- Several gene segments in the Ig gene in the production of single Ig H and L chain
- The gene segments are present in germ cell DNA – transcription and translation only after rearrangement – B cell maturation in BM
- Rearrangement – well regulated process – can generate $>10^8$ specificities at a time – after completion a mature B cell committed to an Ag determinant/epitope

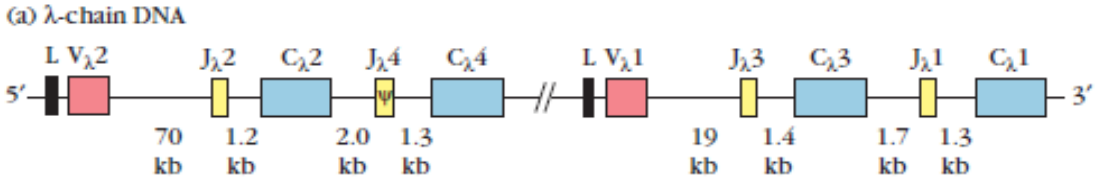
Multigene Organization of Ig Genes

- The κ and λ light chains and the heavy chains are encoded by separate multigene families situated on different chromosomes
- In germ-line DNA, each of these multigene families contains several coding sequences, called **gene segments,** separated by noncoding regions
- During B-cell maturation, these gene segments are rearranged and brought together to form functional immunoglobulin genes
- The κ and λ light-chain families contain **V, J, and C gene segments;** the rearranged VJ segments encode the variable region of the light chains
- The heavy-chain family contains **V, D, J, and C gene segments; the rearranged VDJ gene segments** encode the variable region of the heavy chain
- In each gene family, C gene segments encode the constant regions. Each V gene segment is preceded at its 5 end by a small exon that encodes a short **signal or leader (L) peptide that guides** the heavy or light chain through the endoplasmic reticulum. The signal peptide is cleaved from the nascent light and heavy chains before assembly of the finished immunoglobulin molecule

TABLE 5-1 Chromosomal locations of immunoglobulin genes in human and mouse

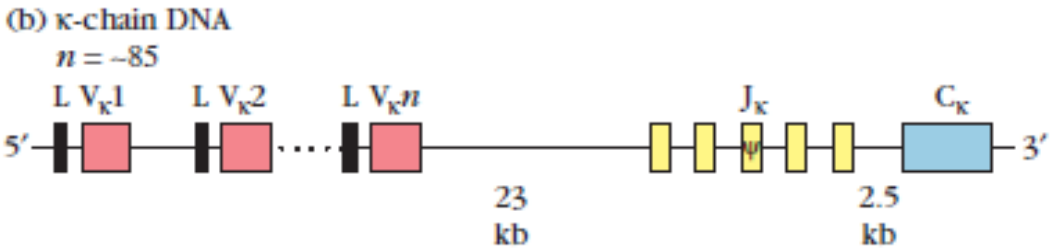
	CHROMOSOME	
Gene	Human	Mouse
λ Light chain	22	16
κ Light chain	2	6
Heavy chain	14	12

V J Arrangement in Light Chain (λ chain)



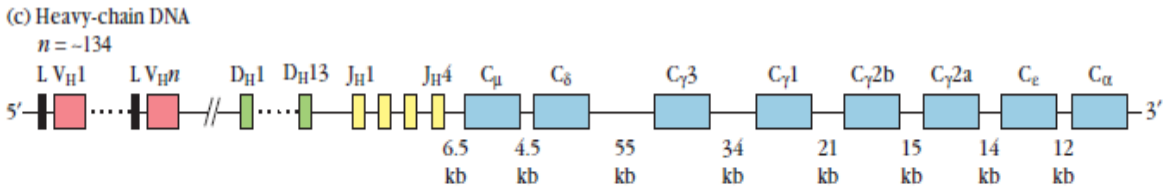
- Mouse germline – 2 V_λ, 4 J_λ and 4 C_λ gene segments
- Functional λ V region gene – 2 coding segments – a 5' V segment and a 3' J segment separated by noncoding DNA in unrearranged Germ Line DNA
- $J_\lambda 4$ & $C_\lambda 4$ – Pseudogenes - No proteins
- V_λ & functional J_λ encode V region
- 3 functional C_λ code for C region – sub types (λ1, λ2, λ3)
- In humans, the lambda locus is more complex – 31 functional V gene segments, 4 J segments, and 7 C segments. In additional to the functional gene segments, the human lambda complex contains many V, J, and C pseudogenes.

V J Arrangement in Light Chain (κ chain)



- Mouse germline – app. 85 V_κ, with adjacent Leader – upstream (5') – 5 J_κ (1 Pseudogene) – 1 C_κ
- V_κ and J_κ – encode Variable region
- C_κ codes for Constant region – only one C gene segment No sub types

V D J Arrangement in heavy Chain

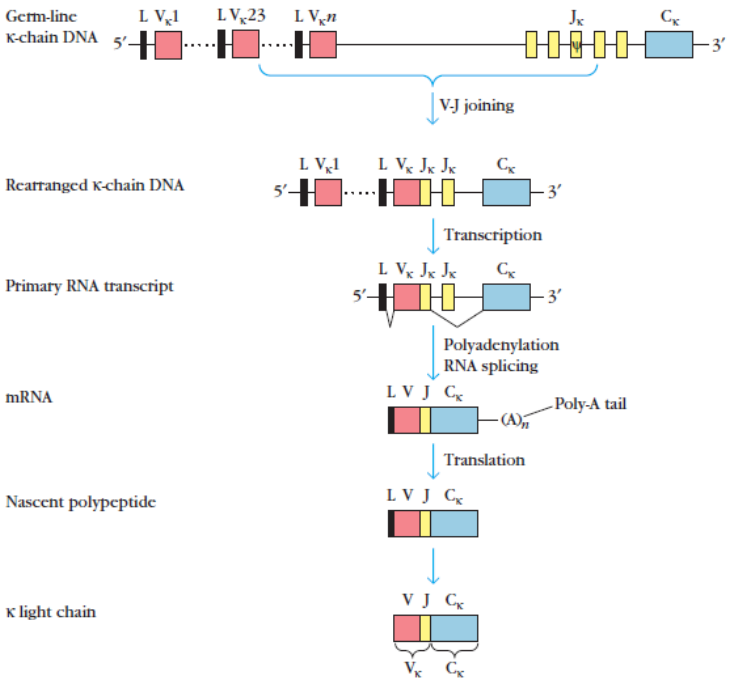


- 3rd Germ Line Gene Segment D (Diversity) joins with V_H and J_H Gene Segments – V region

- D contributes to the generation of Diversity
- In Human,51 V_H upstream to 27 functional D_H
- Each V_H– Leader sequence
- Downstream D_H – 6 functional J_H – followed by a series of C_H gene segments
- Each C_H encodes – C region of heavy chain
- In Humans and mice, C gene segments – sequentially arranged – C_μ, C_δ, C_γ, C_ε, C_α

Variable region gene rearrangements

VJ rearrangements in Light chain DNA



· Multiple IGKV genes for the variable region, V (76 genes, of which 31 to 35 are functional); 5 IGKJ genes for the junctional region, J; a single IGKC gene for the constant region, C; the V, J and C genes are separated in the DNA of the genome ('germline' configuration of the Ig genes).

· These are multigene families (also see the section on the families of genes, in Globin genes "... of the duplications of the ancestor gene have succeeded each other, and the mutations of each of the genes have led to some degree of diversity. Many of these duplicated genes are functional ...").

· First the DNA is rearranged: this makes it possible to join 1 V and 1 J; the intermediate sequences are then deleted,

· The pre-messenger RNA is copied (transcription); this includes introns,

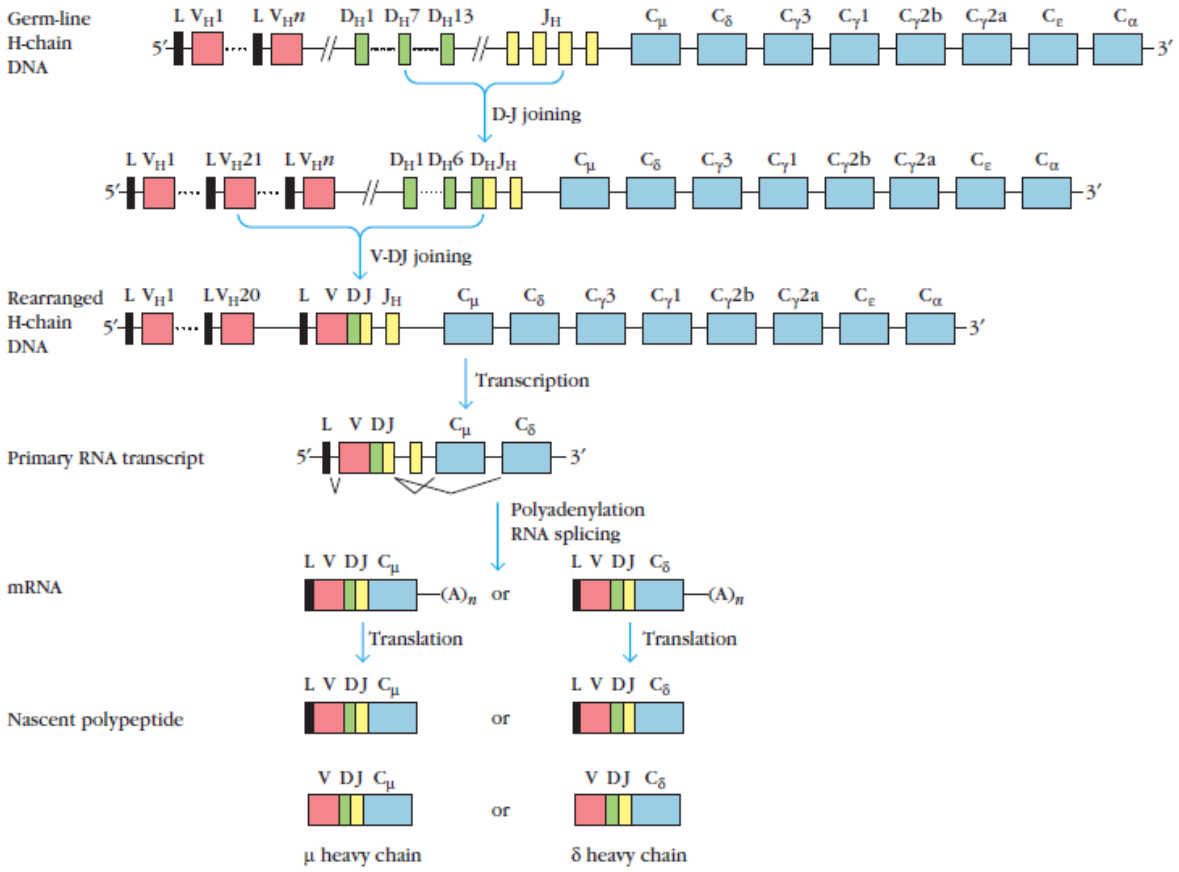
· Then comes splicing: the elimination of the introns from the pre-messenger RNA , to yield mature, messenger RNA,

· This is followed by protein synthesis (known as 'translation').

· N.B.: It is crucial not to confuse DNA **rearrangements** with RNA **splicing**.

NOTE: Only the genes for the immunoglobulins and T-receptors undergo DNA rearrangement.

VDJ rearrangements in Heavy chain DNA



· **II.2. Heavy chains**

· · · IGH ('heavy') genes at 14q32 on chromosome 14.

· · There are 11 IGHC genes, 9 of which are functional (IGHM, IGHD, IGHG1, IGHG2, IGHG3, IGHG4, IGHA1, IGHA2 and IGHE) and correspond respectively to 9 heavy chain isotypes m, d, g1, g2, g3, g4, a1, a2 and e.

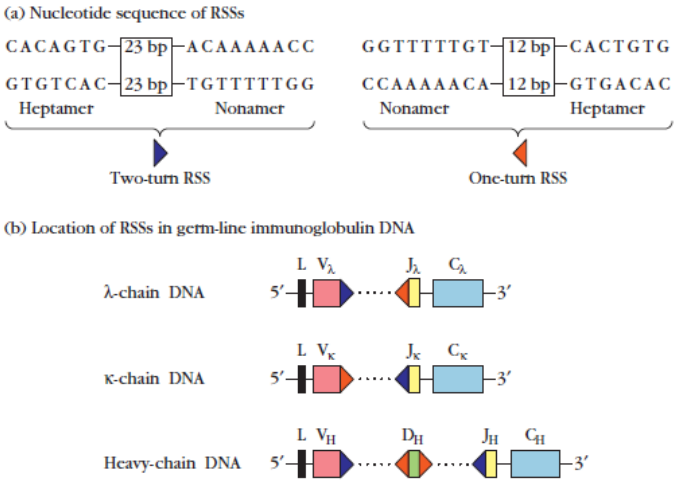
· **II.2.1. V-D-J rearrangements**

· · · DNA rearrangements between one of the 38 to 46 functional variable IGHV genes, one of the 23 functional diversity IGHD genes, and one of the 6 functional junction IGHJ genes:

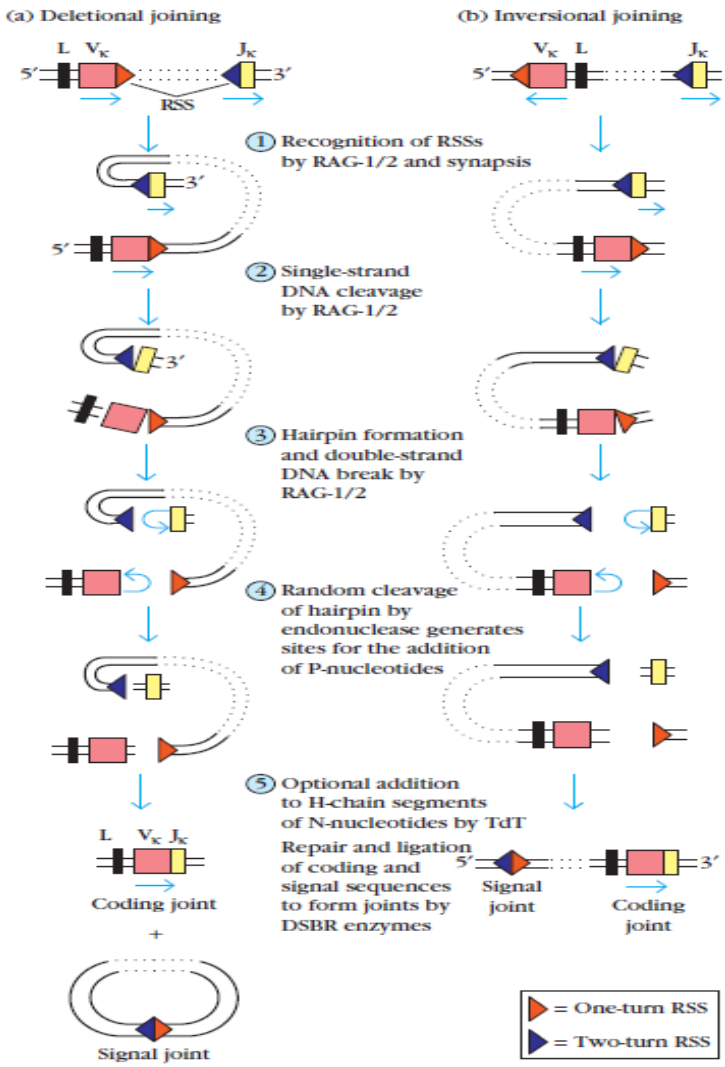
there are also some RSs, which are located downstream (in position 3') of the V genes, either side of the D genes and upstream (at 5') of the J genes. During V-D-J rearrangement, a junction is first formed between 1 D and 1 J, and then one between 1 V and the D-J complex.

Note: there are also 2 or 3 open reading frames for the D genes; each of which can code for 2 or 3 different peptide sequences. The V-D-J junctions are also characterized by nucleotide deletions (by an exonuclease) and by the random addition of nucleotides (by means of TdT, terminal deoxynucleotidyl transferase); **the V regions which result are not, therefore, coded in the genome of the individual and considerably increase the diversity of the V-D-J junctions of the variable domains of the heavy chains of the immunoglobulins.**

Mechanism of Variable-Region DNA Rearrangements



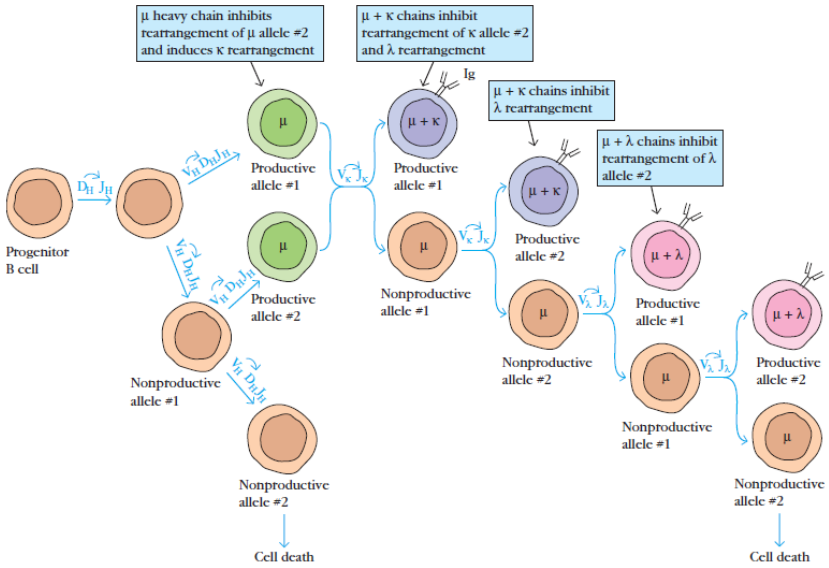
Enzymatic joining of Gene Segment



Generation of Antibody Diversity

- Multiple germ-line gene segments
- Combinatorial V-(D)-J joining
- Junctional flexibility
- P-region nucleotide addition (P-addition)
- N-region nucleotide addition (N-addition)
- Somatic hypermutation
- Combinatorial association of light and heavy chains

Allele exclusion and isotype



· Allele exclusion can be explained in part by the timing of rearrangements, and partly by the surface expression of a functional immunoglobulin, which inhibits the rearrangements and therefore the expression of a second chain. Only one 14 chromosome and one 2 (or 22) chromosome are therefore productive (answer to question D).

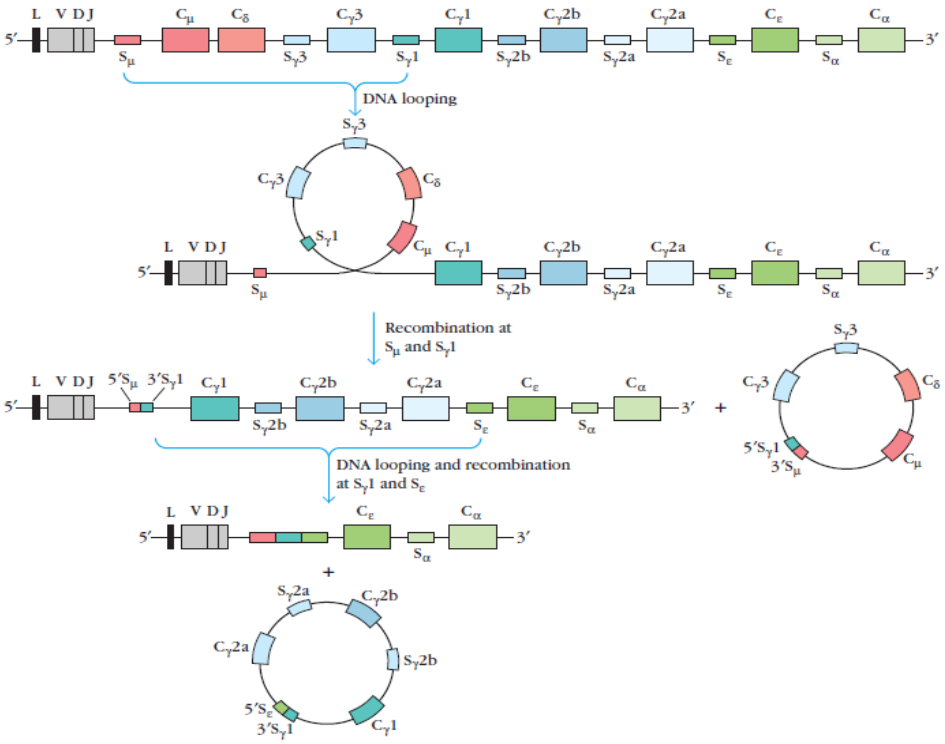
· **II.2.2. Isotype switching**

· · · In the pre-B lymphocyte, a mu chain is first synthesized, because the constant IGHM gene (C) is located near to the V-D-J rearrangement. This mu chain is associated with the pseudo-light chain and the combination constitutes the pre-B receptor. The first complete Ig synthesized by the B-lymphocyte is an IgM, in which the mu chain is combined with a light kappa or lambda chain.

· · During its differentiation, the B lymphocyte can express some other isotype or sub-isotype of Ig. This involves the replacement of an IGHC gene by another, as the result of DNA recombination (isotype switch), with the excision of the entire intermediate part of a deletion loop. This excision occurs at the switch sequences (role related to that of the RSs).

· · The usual sequence is then as follows: synthesis of pre-messenger RNA, splicing of the introns, resulting in mature RNA, and then protein synthesis.

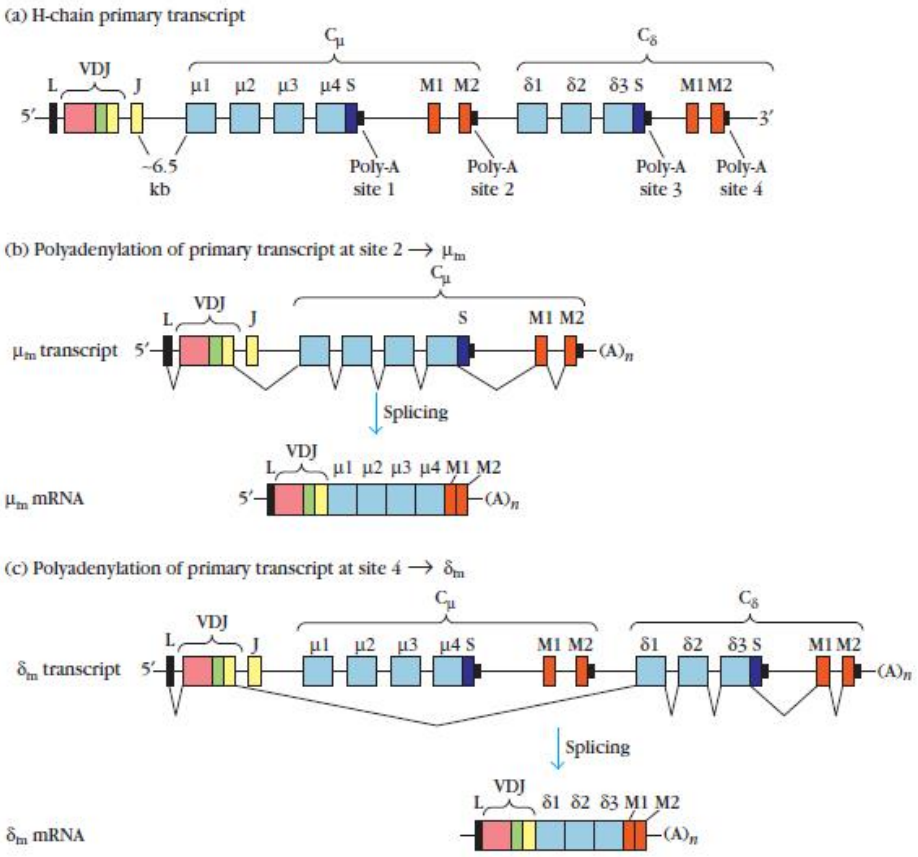
· · This explains why 1) a B-lymphocyte can at first synthesize an IgM and then, during its differentiation, an IgG (IgG1, IgG2, IgG3 or IgG4), an IgA (IgA1 or IgA2) or an IgE, and 2) that it retains the same V-D-J rearrangement and therefore the same antigen recognition site (idiotype) (answer to question C).



· ·

· **I.3. Membrane and secreted Igs**

· · Alternative splicing of the pre-messenger RNA of the heavy chain can yield either a membrane heavy chain (membrane Ig of B lymphocytes), or a secreted heavy chain (plasmocyte secreted Ig), which retain the same V-D-J rearrangement (idiotype) and the same constant region (isotype) (answer to question B).



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· · · Note: the same mechanism (alternative splicing of a pre-messenger) expresses the IgMs and IgDs in the same B cell (situation in mature B cells leaving the bone marrow and reach the lymph nodes via the circulation).

· **III. Conclusions**

· **III.1. Germline diversity: multigene families**

· · · 'Germline' diversity depends on the number of genes at each locus. These are families of genes, offering the possibility of a choice between similar? functional sequences. Possible intergene recombinations permit the long-term evolution of the locus with duplication or deletion of the genes.

· · These genes undergo intragene conversions and recombinations, leading to mixing and diversity (polymorphism) between individuals.

· · The presence of several open reading frames, in the case of IGHD genes, further increases the possibility of choice between similar functional sequences.

· **III.2. Diversity due to DNA rearrangements**

· · · Combination diversity - in the mathematical sense of the term - permits the potential synthesis of a million immunoglobulins. The IGH genes permit the synthesis of about 6000

heavy chains, the IGK or IGL genes of about 160 light chains, which is equivalent to about a million possible combinations 6 x 10 ^{3} x 160).

Locus	Number of functional genes V	D	J	C	Total	Combination diversity V-J or V-D-J
IGH 14q32	38-46	23	6	9	76-84	38 x 23 x 6 = 5244 (min) 46 X 23 X 6 = 6348 (max)
IGK 2p11	31-35	0	5	1	37-41	31 x 5 = 155 (min) 35 x 5 = 175 (max)
IGL 22q11	29-33	0	4-5	4-5	37-43	29 x 4 = 116 (min) 33 x 5 = 165 (max)
					= 150-168	

· · · · In addition to this, during the rearrangements of the IGH of the heavy chains, the acquisition of the N regions, and using one or other of the reading frames for the D genes at the V-D-J junctions, and during the IGK or IGL rearrangements of the light chains, flexibility of the V-J junctions. These mechanisms contribute to increasing the diversity by a factor 10^3 to 10^4 (potential synthesis of 10^9 Ig chains).

· **III.3.Diversity as a result of somatic hypermutations**

· Finally, somatic mutations are extremely numerous (somatic hypermutations) and produce very targeted characterization of the rearranged V-J and V-D-J genes of the Ig, but their mechanism of onset is not yet known. AID (activation-induced cytidine deaminase) may be implicated both in the occurrence of the mutations and the switch mechanism. The mutations appear during the differentiation of the B lymphocyte in the lymph glands and contribute to increasing the diversity of the Igs by a further factor of 10^3, which makes it possible to achieve a potential diversity of 10^{12} different Igs (answer to question A).

· These different mechanisms of diversity make it possible to obtain 10^{12} different immunoglobulins, capable of responding to the several million known antigens (answer to question A).

· The number of different Igs is in fact limited by the number of B cells in a given species.

Complement Pathways: Types, Functions and Regulation

he complement system is a part of the immune system, consists of a series of proteins that interact with one another in a highly regulated manner, in order to eliminate pathogens. It helps antibodies and phagocytic cells to clear pathogens and damaged cells; promote inflammation and attack pathogen's plasma membrane. Proteins that take part in the complement system are called complements that collectively work as a **biological cascade**; the sequence of reactions, each being the catalyst for the next.

Jules Bordet (1895) identified complements as heat-sensitive components in the blood, bearing non-specific antimicrobial activity.

Complements are soluble proteins and glycoproteins mostly **produced by hepatocytes**. More than 20 types of complements are present in serum, found circulating normally in human body in inactive forms *(called as zymogens or proenzymes)*. Complement activation is triggered by an antibody when it is bound to the antigen. It can also be triggered by some components of innate immunity. Thus the **complement system works in both innate and acquired immunity.**

Complements are activated only during inflammatory reactions. During the inflammation, more amount of complements reaches to the interstitial area of the infected tissue through dilated blood vessels, which are then activated by proteolytic cleavage; this exposes the active site of the complements.

Complements are mainly **denoted by the capital letter *C*** with numbers; like, C1, C2, C3, and so on. Some have only alphabet, like, *B, D.* Some are simply represented by names, like, homologous restriction factor.

C1 has three sub-units; C1q, C1r and C1s. C2-C5 have two components, *a* and *b*. **Larger subunits are denoted by *b*** whereas the **smaller are denoted by *a*** *(except C2a, which is larger than C2b).*

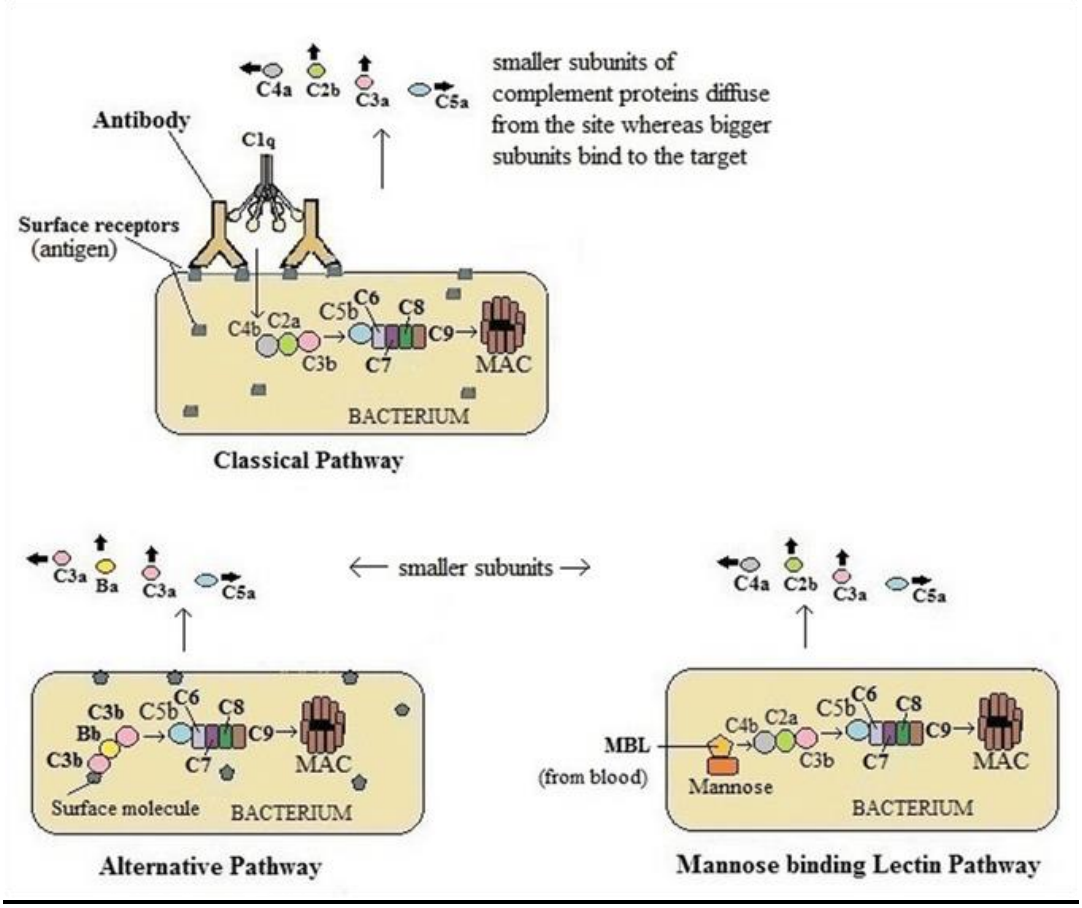
Complement Activation and cell lysis

The complement activation occurs via three pathways; which are:

1. **Classical pathway**
2. **Alternative pathway**
3. **Lectin pathway (or mannose binding lectin pathway)**

The early step of complement system varies in different pathways. However, all the pathways form enzyme complexes; C3 convertase, which cleaves C3 into C3a and C3b; and the C5 convertase, which cleaves C5 into C5a and C5b.C3b, thus formed, binds C3 convertase to form C5 convertase.

C5 convertase, generated by the alternative, classical, or lectin pathway, initiates the activation of late components of the complement system to form membrane attack complex (MAC) and ultimately kills the pathogen.



This occurs through three pathways; **Classical pathway**, activated by antigen-antibody reaction, **Alternative pathway**, activated on microbial cell surfaces, and **Mannose binding Lectin pathway**, activated by a plasma lectin that binds to mannose residues on microbes.

1. **Classical Pathway**

The classical pathway begins with the formation of antigen-antibody complex (immune complex). When an antigen enters the body, the antibody (IgM/IgG) binds to it. This induces conformational changes in the Fc portion of the antibody which exposes a binding site for C1 protein. Hence, the antibody activates the complement system only when bound to an antigen.

C1 is a large, multimeric, protein complex composed of one molecule of C1q and two molecules each of C1r and C1s subunits. C1q binds to the antigen bound antibody (Fc portion). C1r and C1s are proteases which help to cleave C4 and C2.

The immune complex bound to C1 calls another protein C4 which is cleaved into C4a and C4b. C4a goes away whereas activated C4b attaches to the target surface near C1q. Now, C4b attracts C2 which is also cleaved into C2a and C2b. C2a binds C4b forming the C4b2a complex

whereas C2b goes away. The active C4bC2a activates C3. The C4b2a complex is also known as **C3 convertase** as this converts C3 into an active form by separating C3a and C3b. One molecule of C4b2a can cleave a large number of C3 molecules. C3b binds to the microbial surface or to the convertase itself.

C3b when binds to C3 convertase forms C4bC2aC3b (**C5 convertase**) which activates C5.

C5 convertase cleaves C5 into C5a and C5b. C5a diffuses away but C5b is stabilized by binding C6. Then C5bC6 binds to C7. C5bC6C7 complex is then inserted into the phospholipid bilayer of the cell membrane which further binds C8. These all (C5b678) activate C9 to form a macromolecular structure called the **membrane attack complex (MAC)**. This makes hole in the bacterium, as a result, the intracellular contents leak out and unwanted substances get in. Thus, the cell cannot maintain its osmotic stability and the lysis occurs by an influx of water and loss of electrolytes.

This is more effective in Gram negative bacteria than in Gram positive bacteria because MAC formation is easy in the outer membrane in Gram negatives whereas it is difficult in the rigid thick layer of peptidoglycan in Gram positives.

Some of the C3b molecules do not associate with C4b2a; instead these molecules coat immune complexes or microbial cell surfaces and work as opsonins. This process is called opsonization in which opsonin molecule binds one side to the particulate matter i.e. in bacteria, tumor cell, RBC and on the other side they bind to the receptor of phagocytic cell(like, neutrophils and macrophages) which enhance the process of phagocytosis.

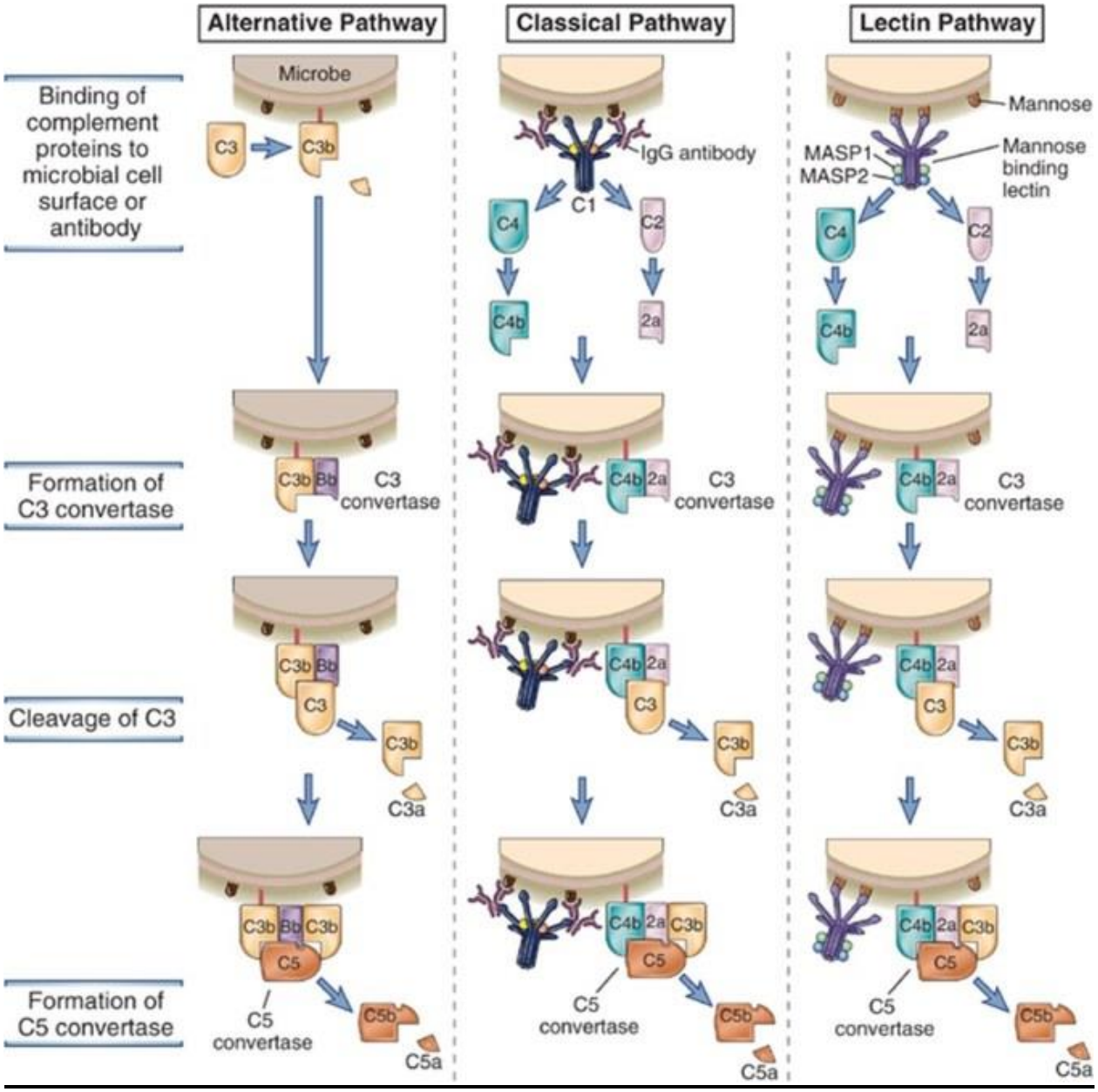
Smaller complement subunits diffuse from the site and can initiate localized inflammatory responses by binding to specific receptors.

1. **Alternative Pathway**

Unlike classical pathway, alternative pathway, does not require Ag-Ab complex for the initiation of complement pathway. It is initiated by cell surface constituents that are foreign to the host. These surface molecules may be **lipopolysaccharide** etc.

When a bacterium enters the host body, as a result of inflammation, complements reach towards the site, where C3 molecules directly touch antigen and become active. In this pathway, serum C3 containing an unstable thioester bond undergoes slow spontaneous hydrolysis to yield C3a and C3b. C3b binds the surface of foreign cell and then binds to another serum protein called factor B. Now the factor B exposes the site which serves as the substrate for enzymatically active serum protein D. Then factor D cleaves B into Ba and Bb forming C3

convertase (C3bBb). C3 convertase then forms C5 convertase which ultimately forms a MAC as in classical pathway.



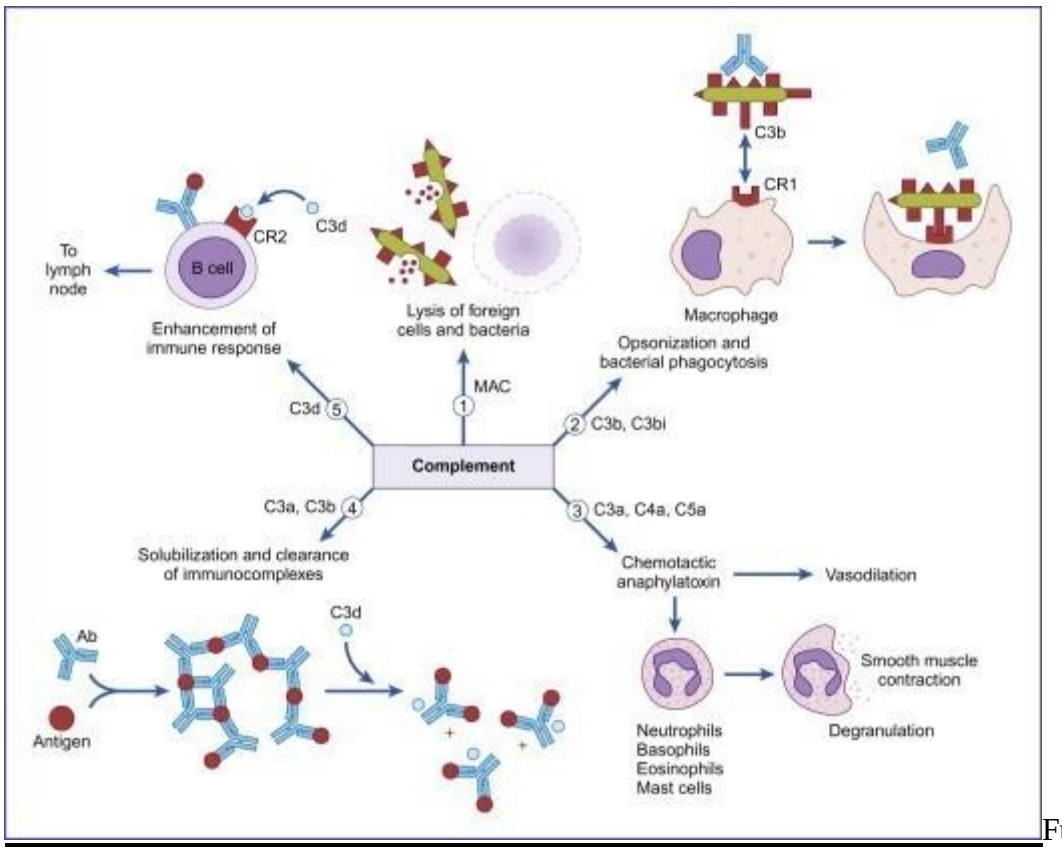
1. **Mannose binding Lectin (MBL) Pathway**

Some bacteria can activate complement system without having antibody and endotoxin. This occurs through MBL pathway which is activated when circulating lectin (MBL) binds to mannose residues on glycoproteins or carbohydrates on the surface of microorganisms. Microorganisms inducing MBL pathway are bacteria, such as Salmonella, Listeria, and Neisseria strains, some fungi and some viruses including HIV-1. MBL is an acute phase protein and its concentration increases during inflammation. The lectin recognizes and binds the carbohydrate of the target cell which then activates complements.

MBL pathway resembles classical pathway as it proceeds through the action of C4 and C2 to produce activated proteins of the complement system. MBL works same as C1q which it resembles in structure.

After the MBL binds to carbohydrate residues on the surface of a cell or pathogen, two components, MASP-1 and MASP-2 bind to MBL. MASP stands for MBL-associated serine proteases. Two proteases form a tetrameric complex similar to the one formed by C1r and C1s and cleaves C4 and C2 forming C3 convertase. The process now continues to form of C5 convertase and the MAC as in classical pathway.

Functions of Complements



Function of Complement Pathway

Some major functions of complements are:

1. **Opsonization and phagocytosis**

C3b, bound to immune complex or coated on the surface of pathogen, activate phagocytic cells. These proteins bind to specific receptors on the phagocytic cells to get engulfed.

1. **Cell lysis**

Membrane attack complex formed by C5b6789 components ruptures the microbial cell surface which kills the cell.

1. **Chemotaxis**

Complement fragments attract neutrophils and macrophages to the area where the antigen is present. These cell surfaces have receptors for complements, like C5a, C3a, thus, run towards the site of inflammation, i.e. chemotaxis.

1. **Activation of mast cells and basophils and enhancement of inflammation**

The proteolytic complement fragments, C5a, C4a, and C3a induce acute inflammation by activating mast cells and neutrophils. All three peptides bind to mast cells and induce degranulation, with the release of vasoactive mediators such as histamine. These peptides are also called anaphylatoxins because the mast cell reactions they trigger are characteristic of anaphylaxis. Binding to specific complement receptors on cells of the immune system, they trigger specific cell functions, inflammation, and secretion of immunoregulatory molecules.

1. **Production of antibodies**

B cells have receptor for C3b. When C3b binds to B-cell, it secretes more antibodies. Thus C3b is also an antibody producing amplifiers which converts it into an effective defense mechanism to destroy invading microorganism.

1. **Immune clearance**

The complement system removes immune complexes from the circulation and deposits them in the spleen and liver. Thus it acts as anti-inflammatory function. Complement proteins promote the solubilization of these complexes and their clearance by phagocytes.

Complement regulation

The complement system has the potential to be extremely damaging to host tissues; hence regulatory mechanisms are required to restrict the complement pathway. Various plasma and cell membrane proteins regulate complement activation by inhibiting different steps in the cascade.

The membrane of most mammalian cells has a high level of sialic acid, which contributes to the inactivation of complements.

Complement related Diseases

Diseases associated with complements can be due to the deficiencies in any of the protein components or in regulatory components.

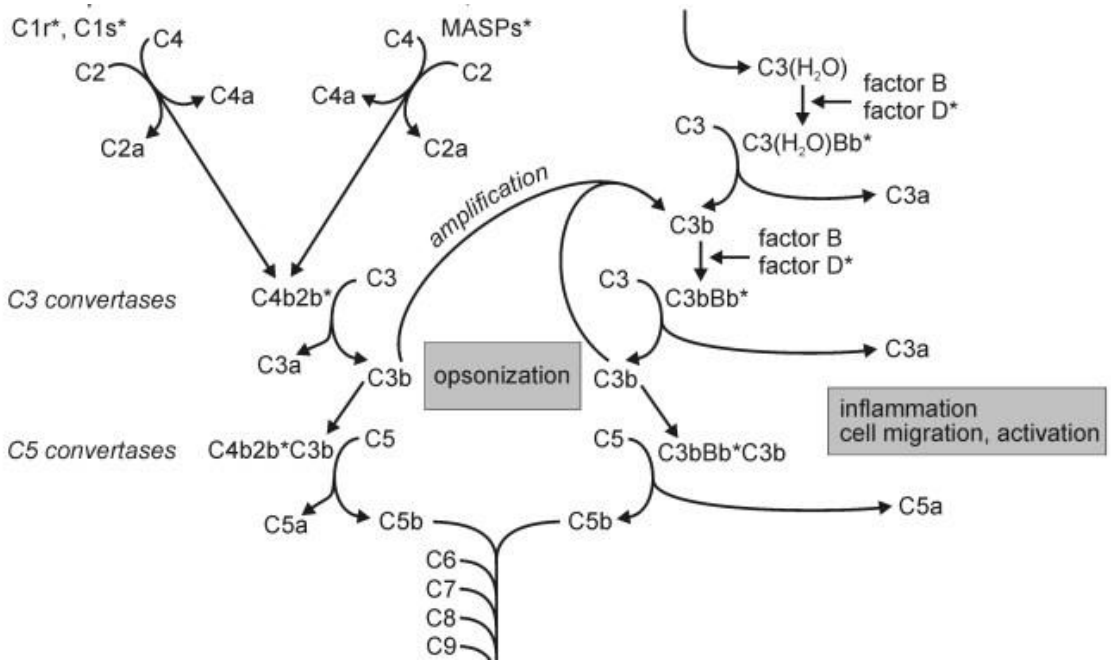
Some examples of complement protein deficiencies are:

Deficiency of C2 and C4 can cause systemic lupus erythematosus; deficiency of C3 and factor D can cause pyogenic bacterial infection; and deficiency of C5-C9 (or MAC deficiency) may lead to the Neisserial infections like, gonorrhea and meningitis.

Deficiencies of regulatory proteins lead to too much activation of complements in wrong time and place which leads to unwanted inflammation and cell lysis. Pyogenic bacterial infection and glomerulonephritis are the results of such deficiencies.

Mutations in the complement regulators factors may lead to atypical hemolytic uremic syndrome, age-related macular degeneration, hereditary angioedema, etc.

Complement system can also be stimulated by abnormal stimuli, like persistent microbes, antibody against self antigens or immune complexes deposited in tissues. Even when the system is properly regulated and activated, it can cause significant tissue damage.



MONOCLONAL ANTIBODY PRODUCTION

Monoclonal antibody (MAb) is a single type of antibody that is directed against a specific antigenic determinant (epitope). It was a dream of scientists to produce MAbs for different antigens. In the early years, animals were immunized against a specific antigen, B-lymphocytes were isolated and cultured in vitro for producing MAbs. This approach was not successful since culturing normal B-lymphocytes is difficult, and the synthesis of MAb was short-lived and very limited.

It is interesting that immortal monoclonal antibody producing cells do exist in nature. They are found in the patients suffering from a disease called multiple myeloma (a cancer of B-lymphocytes). It was in 1975. George Kohler and Cesar Milstein (Nobel Prize, 1984) achieved large scale production of MAbs. They could successfully hybridize antibody—producing B-lymphocytes with myeloma cells in vitro and create a hybridoma.

The result is that the artificially immortalized B-lymphocytes can multiply indefinitely in vitro and produce MAbs. The hybridoma cells possess the growth and multiplying properties of myeloma cells but secrete antibody of B-lymphocytes. The production of monoclonal antibodies by the hybrid cells is referred to as hybridoma technology.

Principle for Creation of Hybridoma Cells:

The myeloma cells used in hybridoma technology must not be capable of synthesizing their own antibodies. The selection of hybridoma cells is based on inhibiting the nucleotide (consequently the DNA) synthesizing machinery. The mammalian cells can synthesize nucleotides by two pathways—de novo synthesis and salvage pathway (Fig. 17.1).

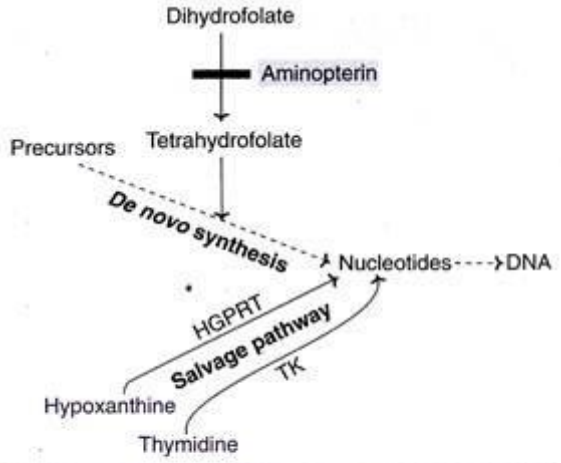


Fig. 17.1 : Pathways for the synthesis of nucleotides (HGPRT–Hypoxanthine guanine phosphoribosyl transferase; TK–Thymidine kinase)

The de novo synthesis of nucleotides requires tetrahydrofolate which is formed from dihydrofolate. The formation of tetrahydrofolate (and therefore nucleotides) can be blocked by the inhibitor aminopterin. The salvage pathway involves the direct conversion of purines and pyrimidine's into the corresponding nucleotides. Hypoxanthine guanine phosphoribosyl transferase (HGPRT) is a key enzyme in the salvage pathway of purines.

It converts hypoxanthine and guanine respectively to inosine monophosphate and guanosine monophosphate. Thymidine kinase (TK), involved in the salvage pathway of pyrimidine's converts thymidine to thymidine monophosphate (TMP). Any mutation in either one of the enzymes (HGPRT or TK) blocks the salvage pathway.

When cells deficient (mutated cells) in HGPRT are grown in a medium containing hypoxanthine aminopterin and Thymidine (HAT medium), they cannot survive due to inhibition of de novo synthesis of purine nucleotides (Note : Salvage pathway is not operative due to lack of HGPRT). Thus, cells lacking HGPRT, grown in HAT medium die.

The hybridoma cells possess the ability of myeloma cells to grow in vitro with a functional HGPRT gene obtained from lymphocytes (with which myeloma cells are fused). Thus, only the hybridoma cells can proliferate in HAT medium, and this procedure is successfully used for their selection.

Production of Monoclonal Antibodies:

The establishment of hybridomas and production of MAbs involves the following steps

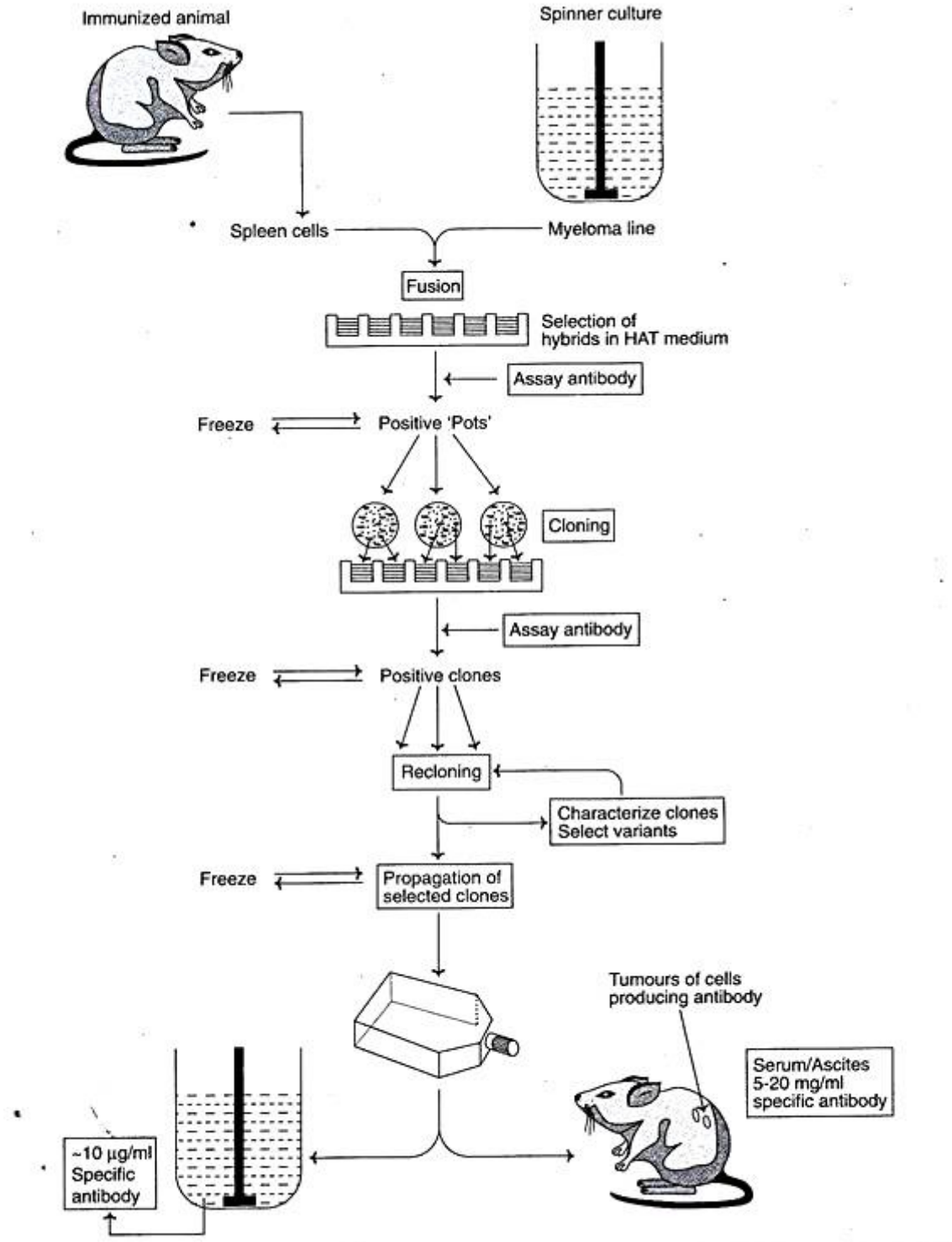


Fig. 17.2 : Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas.

1. Immunization
2. Cell fusion
3. Selection of hybridomas
4. Screening the products

5. Cloning and propagation

6. Characterization and storage.

1. Immunization:

The very first step in hybridoma technology is to immunize an animal (usually a mouse), with appropriate antigen. The antigen, along with an adjuvant like Freund's complete or incomplete adjuvant is injected subcutaneously (adjuvants are non-specific potentiators of specific immune responses). The injections at multiple sites are repeated several times.

This enables increased stimulation of B-lymphocytes which are responding to the antigen. Three days prior to killing of the animal, a final dose of antigen is intravenously administered. The immune-stimulated cells for synthesis of antibodies have grown maximally by this approach. The concentration of the desired antibodies is assayed in the serum of the animal at frequent intervals during the course of immunization.

When the serum concentration of the antibodies is optimal, the animal is sacrificed. The spleen is aseptically removed and disrupted by mechanical or enzymatic methods to release the cells. The lymphocytes of the spleen are separated from the rest of the cells by density gradient centrifugation.

2. Cell Fusion:

The thoroughly washed lymphocytes are mixed with HGPRT defective myeloma cells. The mixture of cells is exposed to polyethylene glycol (PEG) for a short period (a few minutes), since it is toxic. PEG is removed by washing and the cells are kept in a fresh medium. These cells are composed of a mixture of hybridomas (fused cells), free myeloma cells and free lymphocytes.

3. Selection of Hybridomas:

When the cells are cultured in HAT medium (the principle described above), only the hybridoma cells grow, while the rest will slowly disappear. This happens in 7-10 days of culture. Selection of a single antibody producing hybrid cells is very important. This is possible if the hybridomas are isolated and grown individually. The suspension of hybridoma cells is so diluted that the individual aliquots contain on an average one cell each. These cells, when grown in a regular culture medium, produce the desired antibody.

4. Screening the Products:

The hybridomas must be screened for the secretion of the antibody of desired specificity. The culture medium from each hybridoma culture is periodically tested for the desired antibody specificity. The two techniques namely ELISA and RIA are commonly used for this purpose.

In both the assays, the antibody binds to the specific antigen (usually coated to plastic plates) and the unbound antibody and other components of the medium can be washed off. Thus, the hybridoma cells producing the desired antibody can be identified by screening. The antibody secreted by the hybrid cells is referred to as monoclonal antibody.

5. Cloning and Propagation:

The single hybrid cells producing the desired antibody are isolated and cloned. Two techniques are commonly employed for cloning hybrid cells-limiting dilution method and soft agar method.

Limiting dilution method:

In this procedure, the suspension of hybridoma cells is serially diluted and the aliquots of each dilution are put into micro culture wells. The dilutions are so made that each aliquot in a well contains only a single hybrid cell. This ensures that the antibody produced is monoclonal.

Soft agar method:

In this technique, the hybridoma cells are cultured in soft agar. It is possible to simultaneously grow many cells in semisolid medium to form colonies. These colonies will be monoclonal in nature. In actual practice, both the above techniques are combined and used for maximal production of MAbs.

6. Characterization and Storage:

The monoclonal antibody has to be subjected to biochemical and biophysical characterization for the desired specificity. It is also important to elucidate the MAb for the immunoglobulin class or sub-class, the epitope for which it is specific and the number of binding sites it possesses.

The stability of the cell lines and the MAbs are important. The cells (and MAbs) must be characterized for their ability to withstand freezing, and thawing. The desired cell lines are frozen in liquid nitrogen at several stages of cloning and culture.

Large Scale Production of MAbs:

The production MAbs in the culture bottles is rather low (5-10 (ig/ml). The yield can be increased by growing the hybrid cells as ascites in the peritoneal cavity of mice. The ascitic fluid contains about 5-20 mg of MAb/ml. This is far superior than the in vitro cultivation techniques.

But collection of MAb from ascitic fluid is associated with the heavy risk of contamination by pathogenic organisms of the animal. In addition, several animals have to be sacrificed to produce MAb. Hence, many workers prefer in vitro techniques rather than the use of animals.

Encapsulated hybridoma cells for commercial production of MAbs:

The yield of MAb production can be substantially increased by increasing the hybridoma cell density in suspension culture. This can be done by encapsulating the hybridomas in alginate gels and using a coating solution containing poly-lysine (Fig. 17.3). These gels allow the nutrients to enter in and antibodies to come out.

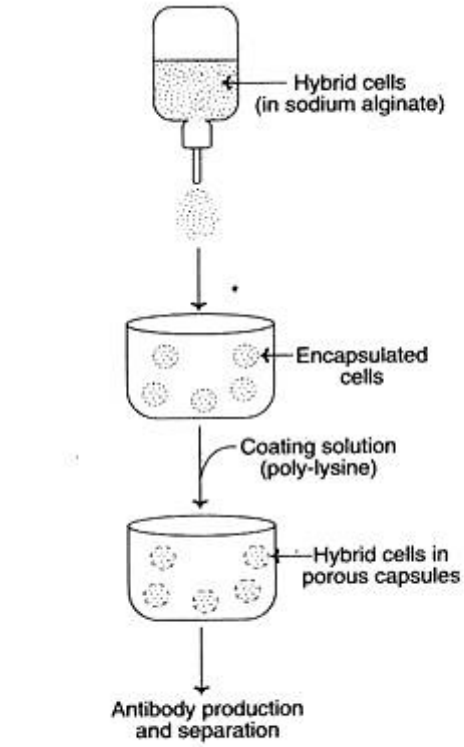


Fig. 17.3 : Production of monoclonal antibodies by microencapsulation.

By this approach, a much higher concentration of MAb production (10-100 µg/ml) can be achieved. Damon Biotech Company and Cell-Tech use encapsulated hybridoma cells for large-scale production of MAbs. They employ 100-liter fermenters to yield about 100g of MAbs in about 2 weeks period.

Human Monoclonal Antibodies:

The monoclonal antibodies produced by using mice are quite suitable for in vitro use. However, their administration to humans is associated with immunological complications, since they are foreign to human body. Production of human monoclonal antibodies is preferred. However, it is difficult to produce human MAbs by conventional hybridoma technology.

The following are the major limitations:

i. For ethical reasons, humans cannot be immunized against antigens.

ii. The fused human lymphocyte-mouse myeloma cells are very unstable.

iii. There are no suitable myeloma cells in humans that can replace mouse myeloma cells.

For the above reasons, alternative arrangements are made to produce human MAbs. These are briefly described below.

Viral transformation of human B-lymphocytes:

B-Lymphocytes, actively synthesizing antibody, are treated with fluorescent-labeled antigen. The fluorescent-activated cells are separated. However, B-cells on their own, cannot grow in culture. This limitation can be overcome by transforming B-lymphocytes with Epstein-Bar virus (EBV). Some of the EBV-transformed cells can grow in culture and produce monoclonal antibodies. Unfortunately, the yield of MAb is very low by this approach.

SCID mouse for producing human MAbs:

The mouse suffering from severe combined immunodeficiency (SCID) disease lacks its natural immunological system. Such mouse can be challenged with appropriate antigens to produce human MAbs.

Transgenic mouse for producing human MAbs:

Attempts have been made in recent years to introduce human immunoglobulin genes into the mice to develop transgenic mice. Such mice are capable of synthesizing human immunoglobulin's when immunized to a particular antigen. The B-lymphocytes isolated from transgenic mice can be used to produce MAbs by the standard hybridoma technology. The above three approaches are quite laborious, and the yield of human MAbs is very low. Consequently, researchers continue their search for better alternatives.

Genetic Engineering Strategies for the Production of Human- Mouse MAbs:

With the advances in genetic engineering, it is now possible to add certain human segments to a mouse antibody. This is truly a hybridized antibody and is referred to as humanized antibody or chimeric antibody.

Substitution of Fv region of human Ig by mouse Fv:

The DNA coding sequences for Fv regions of both L and H chains of human immunoglobulin are replaced by Fv DNA sequence (for L and H chains) from a mouse monoclonal antibody (Fig. 17.4A). The newly developed humanized MAb has Fc region of Ig being human. This stimulates proper immunological response. The chimeric antibodies produced in this manner were found to be effective for the destruction of tumor cells in vitro.

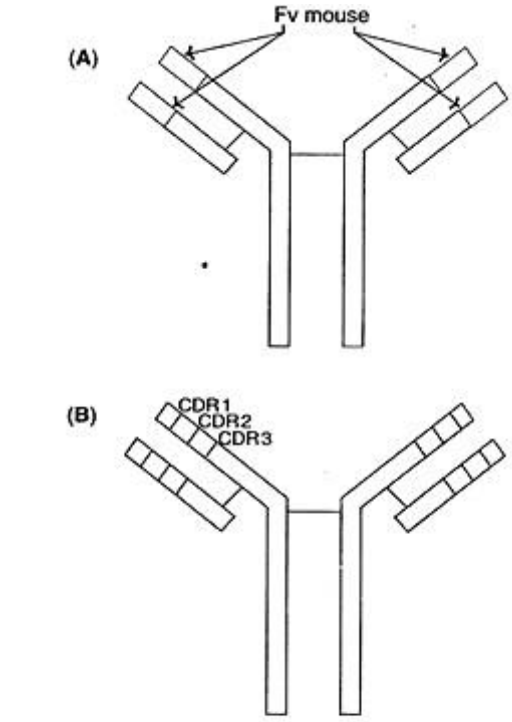


Fig. 17.4 : *Genetically engineered human-mouse antibodies* ***(A)*** *Substitution of Fv region of human Ig by mouse Fv* ***(B)*** *Substitution of human Ig by mouse CDRs (CDR-Complementary determining regions).*

Substitution of Human Ig by Mouse CDRs:

Genetic engineers have been successful in developing human MAbs containing mouse complementary determining regions (CDRs). This is made possible by replacing CDRs genes (CDR1, CDR2, and CDR3) of humans by that of mouse. These chimeric antibodies (Fig. 17.4B) possess the antigen binding affinities of the mouse and they can serve as effective therapeutic agents. So far, about 50 monoclonal antibodies have been produced by this approach. However, this technique is costly and time consuming.

Bi-specific monoclonal antibodies:

The MAbs in which the two arms of Fab (antigen-binding) have two different specificities for two different epitopes are referred to as bi-specific MAbs. They may be produced by fusing two different hybridoma cell lines (Fig. 17.5) or by genetic engineering. Bi-specific Fab MAbs theoretically, are useful for a simultaneous and combined treatment of two different diseases.

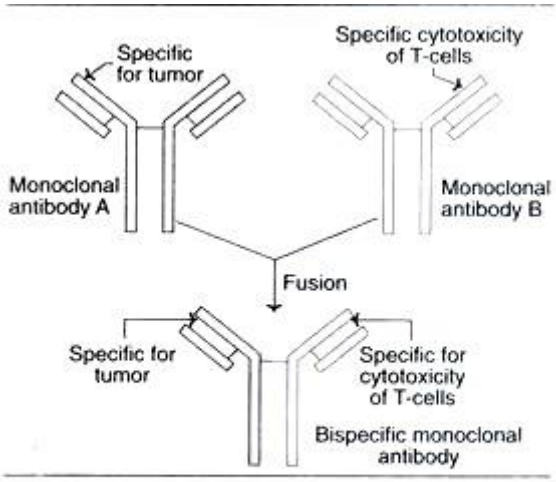


***Fig. 17.5** : Production of a bispecific monoclonal antibody.*

Production of Mabs in E. coli:

The hybridoma technology is very laborious, expensive and time consuming. To overcome these limitation, researchers have been trying to genetically engineer bacteria, plants and animals. The objective is to develop bioreactors for the large scale production of monoclonal antibodies.

It may be noted that the antigen binding regions of antibody (Fv or Fab fragments) are very crucial, while the Fc portion is dispensable. A schematic representation of the procedure adopted for the production of functional antibody fragments is shown in Fig. 17.6, and is briefly described.

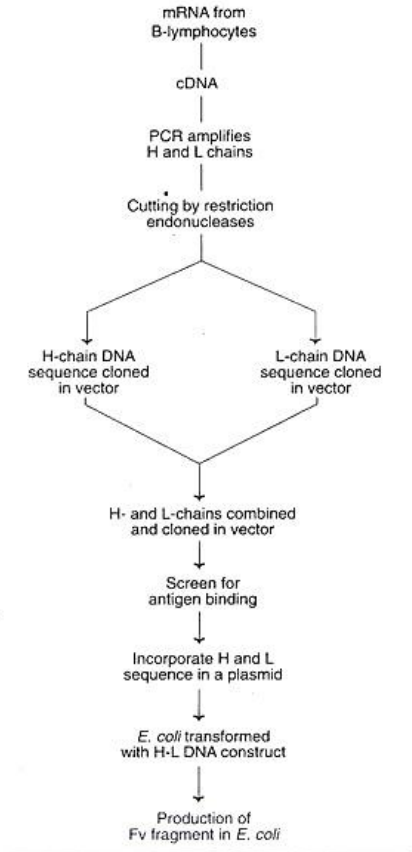


Fig. 17.6 : Production of monoclonal antibodies in E. coli.

The mRNA from isolated B-lymphocytes of either human or mouse is converted to cDNA. The H and L chain sequences of this cDNA are amplified by PCR. The so produced cDNAs are then cut by restriction endonucleases. H and L chain sequences are separately cloned in bacteriophage vectors.

These sequences are put together and cloned in another bacteriophage vector. The combined H and L chains (forming Fv fragment) are screened for antigen binding activity. The specific H and L chains forming a part of the plasmid are transformed in E. coli. These E. coli, in turn, can be harvested to produce Fv fragments to bind to specific antigens.

Second Generation Monoclonal Antibodies:

In the recent years, a number of improvements have been made to produce more specific, sensitive and desired MAbs. This has been possible due to the rapid advances made in genetic engineering techniques. For instances, by employing site- directed mutagenesis, it is possible to introduce cysteine residues at the predetermined positions on the MAb. These cysteine residues which facilitate the isotope labeling may be more useful in diagnostic imaging and radio-immunotherapy.

Advantages of Monoclonal Antibodies:

Monoclonal antibodies truly represent a homogeneous state of a single molecular species. Each MAb is specific to a given antigenic determinant. This is in contrast to the conventional antiserum that contains polyclonal antibodies. The wide range of applications of MAbs is described later.

Limitations of Monoclonal Antibodies:

Hybridoma technology is laborious and time consuming. MAbs are produced against a single antigenic determinant; therefore, they cannot differentiate the molecule as a whole. Sometimes, they may be incapable of distinguishing groups of different molecules also.

The presence of retroviruses as a part of the mammalian chromosomes is a common occurrence. Mice used in MAb production carry several viruses (adenovirus, hepatic virus, retrovirus, reovirus, cytomegalovirus, thymic virus). The presence of some of these viruses has been detected in the hybridomas.

This poses a great danger, since there is no guarantee that MAb produced is totally virus-free, despite the purification. For this reason, US Food and Drug Administration insists that MAb for human use should be totally free from all pathogenic organisms, including viruses.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – V – Immunological Disorders – SBTA1402

HYPERSENSITIVITY

- Generally the immune system is protective
- Protective mechanisms may result in severe damages to tissues and may lead to death

When?

Severe damages may occur when the immune system responded in exaggerated or inappropriate form.

Classification

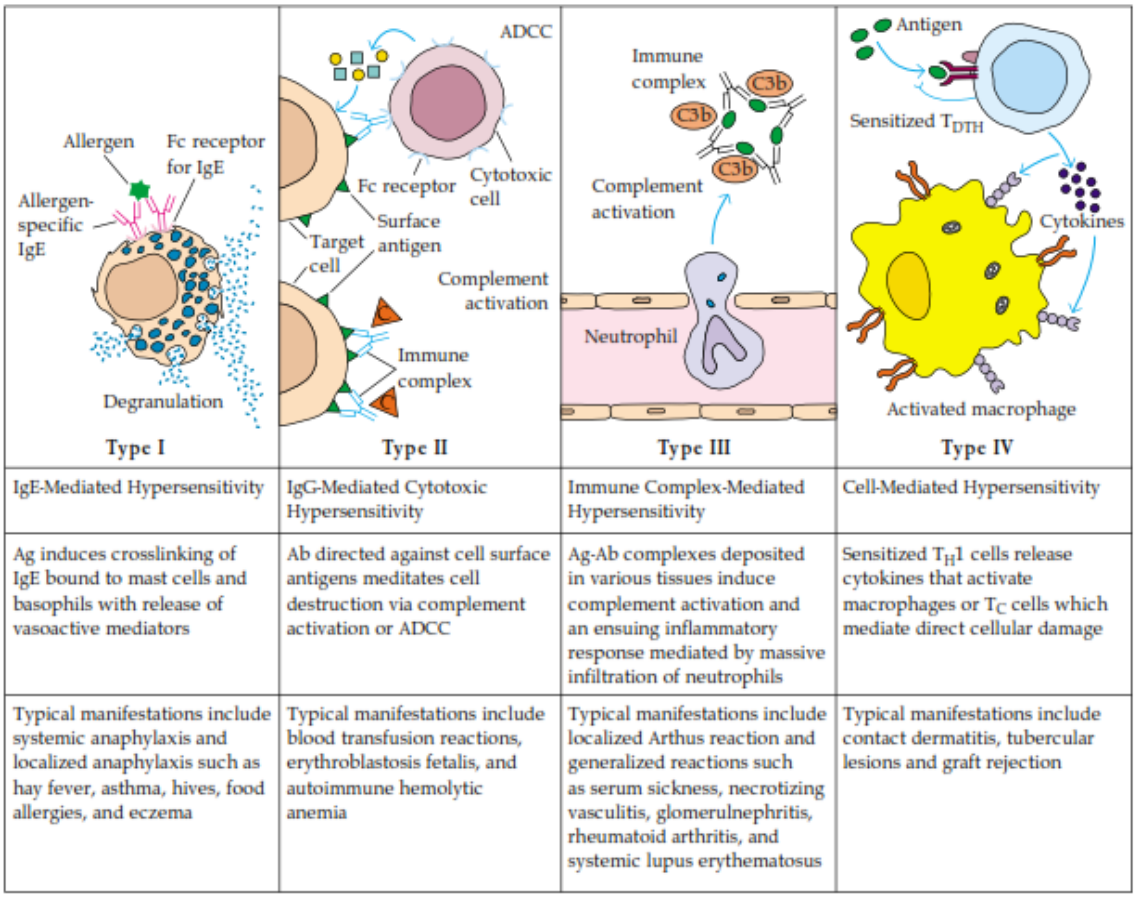
Coombs and Gell classification

1-Type I - immediate (atopic, or anaphylactic)

2-Type II - antibody-dependent

3-Type III - immune complex

4-Type IV - cell-mediated or delayed



Type I	Type II	Type III	Type IV
IgE-Mediated Hypersensitivity	IgG-Mediated Cytotoxic Hypersensitivity	Immune Complex-Mediated Hypersensitivity	Cell-Mediated Hypersensitivity
Ag induces crosslinking of IgE bound to mast cells and basophils with release of vasoactive mediators	Ab directed against cell surface antigens meditates cell destruction via complement activation or ADCC	Ag-Ab complexes deposited in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils	Sensitized T_H1 cells release cytokines that activate macrophages or T_C cells which mediate direct cellular damage
Typical manifestations include systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, food allergies, and eczema	Typical manifestations include blood transfusion reactions, erythroblastosis fetalis, and autoimmune hemolytic anemia	Typical manifestations include localized Arthus reaction and generalized reactions such as serum sickness, necrotizing vasculitis, glomerulnephritis, rheumatoid arthritis, and systemic lupus erythematosus	Typical manifestations include contact dermatitis, tubercular lesions and graft rejection

TYPE I HYPERSENSITIVITY

Type I - immediate (or atopic, or anaphylactic)

- Type I hypersensitivity is an allergic reaction provoked by re-exposure to a specific antigen**.**
- Exposure may be by ingestion**,** inhalation**,** injection, or direct contact.
- The reaction is mediated by IgE antibodies and produced by the immediate release of histamine**,** tryptase**,** arachidonate and derivatives by basophils and mast cells**..**
- This causes an inflammatory response leading to an immediate (within seconds to minutes) reaction.
- The reaction may be either local or systemic. Symptoms vary from mild irritation to sudden death from anaphylactic shock.
- Treatment usually involves epinephrine**,** antihistamines, and corticosteroids
- Type I hypersensitivity is also known as **immediate** or anaphylactic hypersensitivity.
- The reaction may involve skin (urticaria and eczema), eyes (conjunctivitis), nasopharynx (rhinorrhea, rhinitis), bronchopulmonary tissues (asthma) and gastrointestinal tract (gastroenteritis)
- Immediate hypersensitivity is mediated by **IgE**.
- The primary cellular component in this hypersensitivity is the **mast cell** or **basophil**.
- The reaction is amplified and/or modified by platelets, neutrophils and eosinophils.
- A biopsy of the reaction site demonstrates mainly **mast cells** and **eosinophils**.

Mechanism of type I hypersensitivity

The biological mediator on effect stage

1. Histamine:

Dilate blood vessel　　Increase vascular permeability

2. Leukotrienes:

Bronchial smooth muscles contract ——— Asthmas

3. Prostaglandin:

High concentration of PGE ——— Inhibit the secretion of histamine

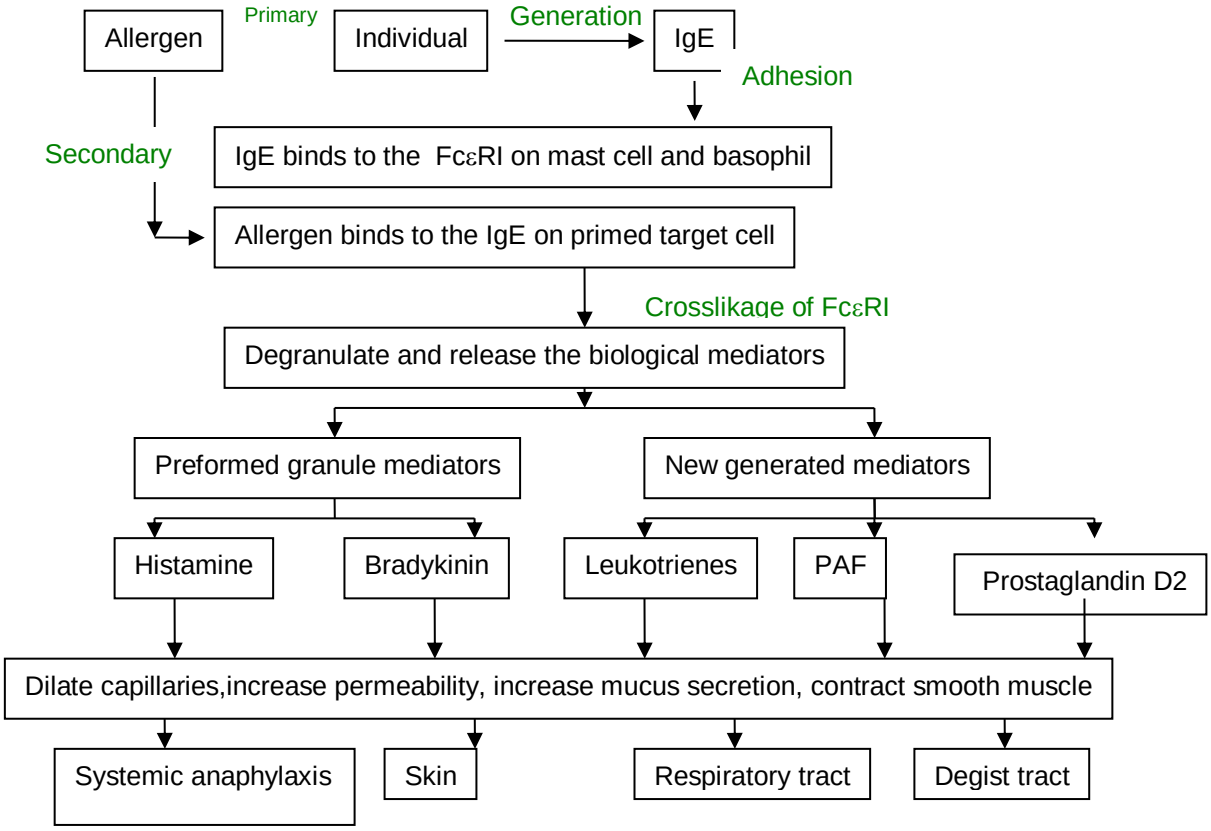
low concentration of PGE ——— promote the release of histamine

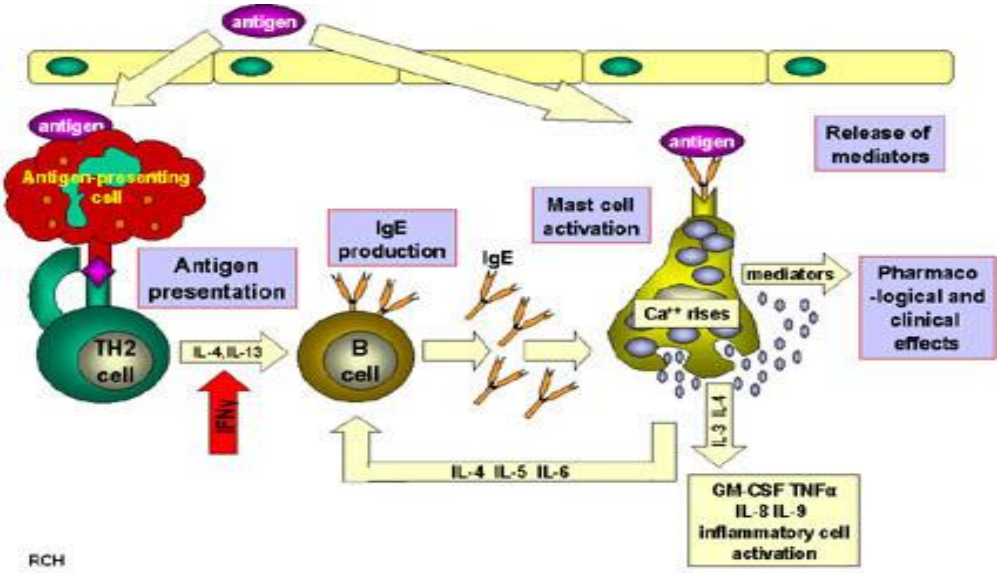
4. Platelet activating factor (PAF) :

Agglutinate and activate platelets to release histamine

5. Eosinophil chemotactic factor（ECF-A）:

6. Bradykinin : Vasodilator function





1. Systemic anaphylaxis: **a very dangerous syndrome**

1) Anaphylactic drug allergy： penicillin

2) Anaphylactic serum allergy：

2. Respiratory allergic diseases :

1) Allergic asthma：acute response, chronic response

2) Allergic rhinitis

3. Gastrointestinal allergic diseases :

The lack of SIgA protein hydrolase ⟶ Undigested protein ⟶ Allergen

4. Skin allergy:

5. Therapy of type I hypersensitivity

1. **Allergen avoidance : Atopy patch test**
2. **Desensitivity therapy / Hyposensitization :**

1) Allogenic serum desensitivity therapy:

Repeated injection small amounts of allergen, Temporality

2) Specific allergen desensitivity therapy

IgG+allergen Neutralizing antibody, Blocking antibody

3. **Drug therapy:**

1) Stabilization of triggering cells

sodium cromoglycate stabilize the membrane, inhibit mast cell degranulation

2) Mediator antagonism

Chlor-Trimeton —— Antihistamine

Acetylsalicylic acid —— Bradykinin antagonism

3) Improve the responsibility of target organs

4. **New immunotherapy :**

Some examples:

- **Allergic** asthma
- **Allergic** conjunctivitis
- Allergic rhinitis **("hay fever")**
- Anaphylaxis
- Angioedema
- Urticaria **(hives**)

TYPE II HYPERSENSITIVITY

Type II - antibody-dependent

- In type II hypersensitivity, the antibodies produced by the immune response bind to antigens on the patient's own cell surfaces.
- The antigens recognized in this way may either be intrinsic ("self" antigen, innately part of the patient's cells) or extrinsic (absorbed onto the cells during exposure to some foreign antigen, possibly as part of infection with a pathogen

2. Mechanism of Type II hypersentivity

1. Surface antigen on target cells

Target cells: Normal tissue cell, changed or modified self tissue cells

Antigen : Blood group antigen, Common antigen, Drug antigen,

Self-antigen modified by physical factors or infection

Antigen-antibody complex

2. Antibody, complement and modified self-cell

Activate complement ——— Lyse target cells

Opsonic phogacytosis ——— Destroy target cells

Mϕ、NK、 T ——— ADCC

Stimulating or blocking effect ——— Promote /suppress the target cell funcion

3. Common disease of type II hypersensitivity

1)Transfusion reaction

hemolysis : mismatch of ABO blood group, severely destroy RBC

nonhemolysis : repeat transfusion of allogenic HLA

drug anaphylactic shock：penicilline

2) Hemolytic disease of newborn

Mother Rh^- : first baby Rh^+ (Ab), second baby Rh^+,

fetal RBC destroyed

3) Autoimmune hemolytic anemia and type II drug reaction

i. Foreign antigen or hapten

Penicillin	**RBC**	**hemolytic anemia**
Quinin	**Platlet**	**thrombocytopenic purpura**
Pyramidone	**Granulocyte**	**agranulocytosis**

ii. Self-antigen

Drug conversion from a hapten to a full antigen

induce self antibody autoimmune hemolytic anemia

4.Anti -glomerular basement membrane nephritis

β-Hemolytic streptococcus and human glomerular basement membrane

Common antigen ---- cross reaction ---nephrotoxic nephritis

5. Super acute rejection in allogenic organ transplantation

6. Goodpasture syndrome

7.Hyperthyroidism or hypothyroidism—receptor diseases

E**xamples**

- Autoimmune haemolytic anaemia
- Pernicious anemia
- Immune thrombocytopenia
- Transfusion reactions
- Hashimoto's thyroiditis
- Graves' disease
- Myasthenia gravis
- Farmer's Lung
- Hemolytic disease of the newborn

TYPE III HYPERSENSITIVITY

- Also known as immune complex disease
- occurs when immune complex (Ag-Ab) are not removed from circulation
- These complexes are deposited in various tissues and organs such as:

- Kidneys
- Joints
- Lung
- Skin

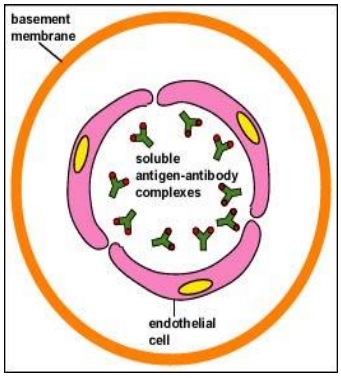
In type III hypersensitivity:

- soluble immune complexes (aggregations of antigens and IgG and IgM antibodies) form in the blood and are deposited in various tissues (typically the skin, kidney and joints)
- This may trigger an immune response according to the classical pathway of complement activation.
- The reaction takes hours to days to develop
- Immune complex formation may occur as a result of :

✓ Autoimmune diseases (RA)
✓ Persistence infection (Hepatitis virus)
✓ Repeated inhalation of antigenic materials

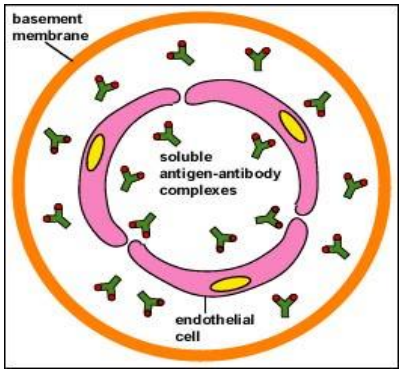
MECHANISM

Step 1



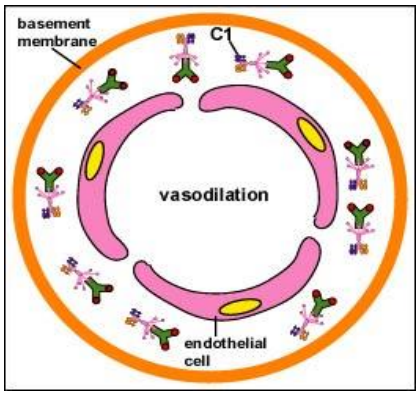
Large quantities of soluble antigen-antibody complexes form in the blood and are not completely removed by macrophages.

Step 2



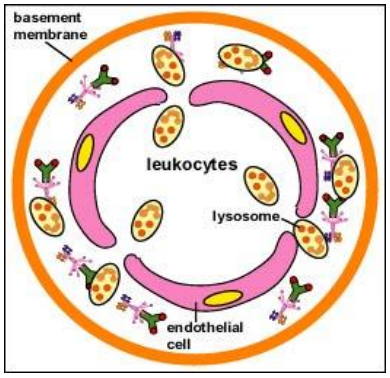
These antigen-antibody complexes lodge in the capillaries between the endothelial cells and the basement membrane.

Step 3



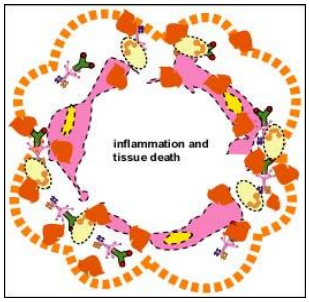
These antigen-antibody complexes activate the classical complement pathway leading to vasodilatation.

Step 4



The complement proteins and antigen-antibody complexes attract leukocytes to the area.

Step 5



The leukocytes discharge their killing agents and promote massive inflammation. This can lead to tissue death and hemorrhage.

3. common disease of type III hypersensitivity

1. Local immune complex disease

Arthus reaction： Experimental local reaction,

Necrotic vasculitis vasculitis, Ulcer

Human local reaction: insulin-dependent diabetes mellitus (IDDM)

2. Acute systemic immune complex disease

serum sickness

Anti-serum Ab+Ag systemic tissue injury ,fever, arthritis, skin rash

Penicillin、Sulfanilamide

Acute immune complex glomerulonephritis : Streptococcus infection

3. Chronic immune complex disease

SLE

Rheumatoid arthritis： RF+IgG Deposit on synovial membrane

Examples:

- Immune complex glomerulonephritis
- Rheumatoid arthritis

- Serum sickness
- Subacute bacterial endocarditis
- Symptoms of malaria
- Systemic lupus erythematosus
- Arthus reaction

TYPE IV HYPERSENSITIVITY

- Type IV hypersensitivity is also known as cell mediated or delayed type hypersensitivity.
- The classical example of this hypersensitivity is tuberculin (Montoux) reaction
- Reaction peaks 48 hours after the injection of antigen (PPD or old tuberculin). The lesion is characterized by induration and erythema
- Type IV hypersensitivity is often called delayed type as the reaction takes two to three days to develop.
- Unlike the other types, it is not antibody mediated but rather is a type of cell-mediated response.
- Another form of delayed hypersensitivity is contact dermatitis (poison ivy (figure 6), chemicals, heavy metals, *etc.*) in which the lesions are more papular
- Type IV hypersensitivity can be classified into three categories depending on the time of onset and clinical and histological presentation

Mechanism:

- The mechanism includes T lymphocytes and monocytes and/or macrophages.
- Cytotoxic T cells (Tc) cause direct damage whereas helper T (TH1) cells secrete cytokines which activate cytotoxic T cells, recruit and activate monocytes and macrophages, which cause the bulk of the damage
- The delayed hypersensitivity lesions mainly contain monocytes and a few T cells.

2. Mechanism of type IV hypersensitivity

Formation of effector and memory T cells

Inflammation and cytotoxicity caused by effector T cells

1) Inflammation and tissue injury mediated by CD4+Th1

Release chemokines and cytokines

Immune injury mainly caused by infiltration of mononuclear cells and lymphocytes

2) Cytotoxicity of CD8+CTL

3. Common disease of type IV hypersensitivity

1) Infectious delayed type hypersensitivity

OT(Old Tuberculin) test

2) Contact dermatitis :

Paint, drug red rash, papula, water blister, dermatitis

3) Acute rejection of allogenic transplantation and

immune response in local tumor mass

Same disease (SLE), multiple immune injury ,hypersensitivity involved

Some clinical examples:

- Contact dermatitis (poison ivy rash, for example)
- Temporal arteritis
- Symptoms of leprosy
- Symptoms of tuberculosis
- Transplant rejection

The hypersensitivity reactions

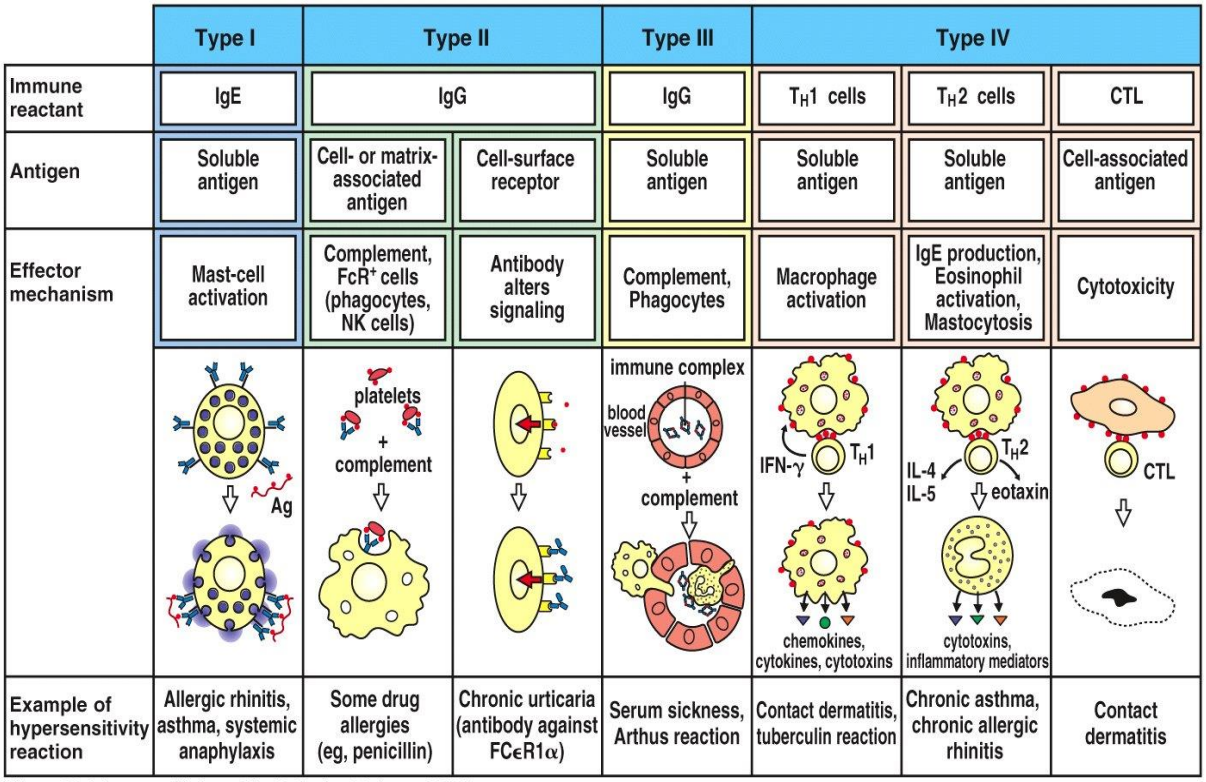
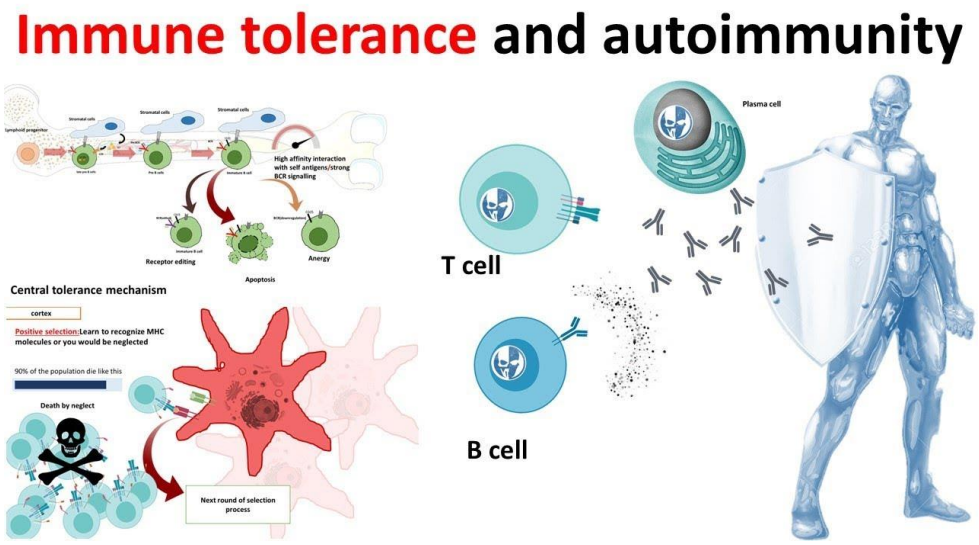
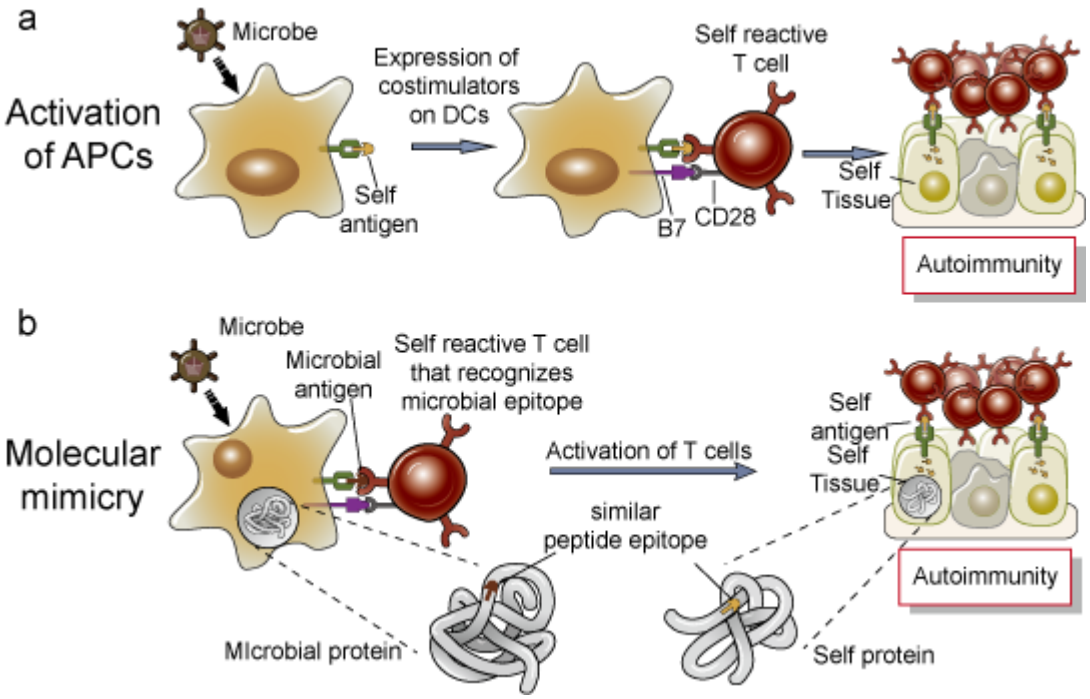


Figure 12-2 Immunobiology, 6/e. (© Garland Science 2005)

IMMUNE TOLERANCE





AUTOIMMUNITY

What is an autoimmune disease?

An autoimmune disease is a condition in which your immune system mistakenly attacks your body.

The immune system normally guards against germs like bacteria and viruses. When it senses these foreign invaders, it sends out an army of fighter cells to attack them.

Normally, the immune system can tell the difference between foreign cells and your own cells.

In an autoimmune disease, the immune system mistakes part of your body, like your joints or skin, as foreign. It releases proteins called autoantibodies that attack healthy cells.

Some autoimmune diseases target only one organ. Type 1 diabetes damages the pancreas. Other diseases, like systemic lupus erythematosus (SLE), affect the whole body.

Why does the immune system attack the body?

Doctors don't know exactly what causes the immune-system misfire. Yet some people are more likely to get an autoimmune disease than others.

According to a 2014 study, women get autoimmune diseases at a rate of about 2 to 1 compared to men — 6.4 percent of women vs. 2.7 percent of men. Often the disease starts during a woman's childbearing years (ages 15 to 44).

Some autoimmune diseases are more common in certain ethnic groups. For example, lupus affects more African-American and Hispanic people than Caucasians.

Certain autoimmune diseases, like multiple sclerosis and lupus, run in families. Not every family member will necessarily have the same disease, but they inherit a susceptibility to an autoimmune condition.

Because the incidence of autoimmune diseases is rising, researchers suspect environmental factors like infections and exposure to chemicals or solvents might also be involved.

A "Western diet" is another suspected risk factor for developing an autoimmune disease. Eating high-fat, high-sugar, and highly processed foods is thought to be linked to inflammation, which might set off an immune response. However, this hasn't been proven.

A 2015 study focused on another theory called the hygiene hypothesis. Because of vaccines and antiseptics, children today aren't exposed to as many germs as they were in the past. The lack of exposure could make their immune system prone to overreact to harmless substances.

BOTTOM LINE: Researchers don't know exactly what causes autoimmune diseases. Genetics, diet, infections, and exposure to chemicals might be involved.

14 common autoimmune diseases

There are more than 80 different autoimmune diseases. Here are 14 of the most common ones.

1. Type 1 diabetes

The pancreas produces the hormone insulin, which helps regulate blood sugar levels. In type 1 diabetes mellitus, the immune system attacks and destroys insulin-producing cells in the pancreas.

High blood sugar results can lead to damage in the blood vessels, as well as organs like the heart, kidneys, eyes, and nerves.

2. Rheumatoid arthritis (RA)

In rheumatoid arthritis (RA), the immune system attacks the joints. This attack causes redness, warmth, soreness, and stiffness in the joints.

Unlike osteoarthritis, which commonly affects people as they get older, RA can start as early as your 30s or sooner.

3. Psoriasis/psoriatic arthritis

Skin cells normally grow and then shed when they're no longer needed. Psoriasis causes skin cells to multiply too quickly. The extra cells build up and form inflamed red patches, commonly with silver-white scales of plaque on the skin.

Up to 30 percent of people with psoriasis also develop swelling, stiffness, and pain in their joints. This form of the disease is called psoriatic arthritis.

4. Multiple sclerosis

Multiple sclerosis (MS) damages the myelin sheath, the protective coating that surrounds nerve cells, in your central nervous system. Damage to the myelin sheath slows the transmission speed of messages between your brain and spinal cord to and from the rest of your body.

This damage can lead to symptoms like numbness, weakness, balance issues, and trouble walking. The disease comes in several forms that progress at different rates. According to a 2012 studyTrusted Source, about 50 percent of people with MS need help walking within 15 years after the disease starts.

5. Systemic lupus erythematosus (SLE)

Although doctors in the 1800s first described lupus as a skin disease because of the rash it commonly produces, the systemic form, which is most the common, actually affects many organs, including the joints, kidneys, brain, and heart.

Joint pain, fatigue, and rashes are among the most common symptoms.

6. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a term used to describe conditions that cause inflammation in the lining of the intestinal wall. Each type of IBD affects a different part of the GI tract.

- Crohn's disease can inflame any part of the GI tract, from the mouth to the anus.
- Ulcerative colitisaffects only the lining of the large intestine (colon) and rectum.

7. Addison's disease

Addison's disease affects the adrenal glands, which produce the hormones cortisol and aldosterone as well as androgen hormones. Having too little of cortisol can affect the way the body uses and stores carbohydrates and sugar (glucose). Deficiency of aldosterone will lead to sodium loss and excess potassium in the bloodstream.

Symptoms include weakness, fatigue, weight loss, and low blood sugar.

8. Graves' disease

Graves' disease attacks the thyroid gland in the neck, causing it to produce too much of its hormones. Thyroid hormones control the body's energy usage, known as metabolism.

Having too much of these hormones revs up your body's activities, causing symptoms like nervousness, a fast heartbeat, heat intolerance, and weight loss.

One potential symptom of this disease is bulging eyes, called exophthalmos. It can occur as a part of what is called Graves' ophthalmopathy, which occurs in around 30 percent of those who have Graves' disease, according to a 1993 studyTrusted Source.

9. Sjögren's syndrome

This condition attacks the glands that provide lubrication to the eyes and mouth. The hallmark symptoms of Sjögren's syndrome are dry eyes and dry mouth, but it may also affect the joints or skin.

10. Hashimoto's thyroiditis

In Hashimoto's thyroiditis, thyroid hormone production slows to a deficiency. Symptoms include weight gain, sensitivity to cold, fatigue, hair loss, and swelling of the thyroid (goiter).

11. Myasthenia gravis

Myasthenia gravis affects nerve impulses that help the brain control the muscles. When the communication from nerves to muscles is impaired, signals can't direct the muscles to contract.

The most common symptom is muscle weakness that gets worse with activity and improves with rest. Often muscles that control eye movements, eyelid opening, swallowing, and facial movements are involved.

12. Autoimmune vasculitis

Autoimmune vasculitis happens when the immune system attacks blood vessels. The inflammation that results narrows the arteries and veins, allowing less blood to flow through them.

13. Pernicious anemia

This condition causes deficiency of a protein, made by stomach lining cells, known as intrinsic factor that is needed in order for the small intestine to absorb vitamin B-12 from food. Without enough of this vitamin, one will develop an anemia, and the body's ability for proper DNA synthesis will be altered.

Pernicious anemia is more common in older adults. According to a 2012 study, it affects 0.1 percent of people in general, but nearly 2 percent of people over age 60.

14. Celiac disease

People with celiac disease can't eat foods containing gluten, a protein found in wheat, rye, and other grain products. When gluten is in the small intestine, the immune system attacks this part of the gastrointestinal tract and causes inflammation.

A 2015 studyTrusted Source noted that celiac disease affects about 1 percent of people in the United States. A larger number of people have reported gluten sensitivity, which isn't an autoimmune disease, but can have similar symptoms like diarrhea and abdominal pain.

Autoimmune disease symptoms

The early symptoms of many autoimmune diseases are very similar, such as:

- fatigue
- achy muscles
- swelling and redness
- low-grade fever
- trouble concentrating
- numbness and tingling in the hands and feet

- hair loss
- skin rashes

Individual diseases can also have their own unique symptoms. For example, type 1 diabetes causes extreme thirst, weight loss, and fatigue. IBD causes belly pain, bloating, and diarrhea.

With autoimmune diseases like psoriasis or RA, symptoms may come and go. A period of symptoms is called a flare-up. A period when the symptoms go away is called remission.

BOTTOM LINE: Symptoms like fatigue, muscle aches, swelling, and redness could be signs of an autoimmune disease. Symptoms might come and go over time.

When to see a doctor

See a doctor if you have symptoms of an autoimmune disease. You might need to visit a specialist, depending on the type of disease you have.

- **Rheumatologists** treat joint diseases, like rheumatoid arthritis as well as other autoimmune diseases like Sjögren's syndrome and SLE.
- **Gastroenterologists** treat diseases of the GI tract, such as celiac and Crohn's disease.
- **Endocrinologists** treat conditions of the glands, including Graves' disease, Hashimoto's thyroiditis, and Addison's disease.
- **Dermatologists** treat skin conditions, such as psoriasis.

Tests that diagnose autoimmune diseases

No single test can diagnose most autoimmune diseases. Your doctor will use a combination of tests and a review of your symptoms and physical examination to diagnose you.

The antinuclear antibody test (ANA) is often one of the first tests that doctors use when symptoms suggest an autoimmune disease. A positive test means you may have one of these diseases, but it won't confirm exactly which one you have or if you have one for sure.

Other tests look for specific autoantibodies produced in certain autoimmune diseases. Your doctor might also do nonspecific tests to check for the inflammation these diseases produce in the body.

BOTTOM LINE: A positive ANA blood test may be indicative of an autoimmune disease. Your doctor can use your symptoms and other tests to confirm the diagnosis.

How are autoimmune diseases treated?

Treatments can't cure autoimmune diseases, but they can control the overactive immune response and bring down inflammation or at least reduce pain and inflammation. Drugs used to treat these conditions include:

- nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen (Motrin, Advil) and naproxen (Naprosyn)

- immune-suppressing drugs

Treatments are also available to relieve symptoms like pain, swelling, fatigue, and skin rashes.

Eating a well-balanced diet and getting regular exercise may also help you feel better.

BOTTOM LINE: The main treatment for autoimmune diseases is with medications that bring down inflammation and calm the overactive immune response. Treatments can also help relieve symptoms.

Immunological Tolerance

- Immunological tolerance is the failure to mount an immune response to an antigen. It can be:
- Natural or "self" tolerance. This is the failure (a good thing) to attack the body's own proteins and other antigens. If the immune system should respond to "self", an autoimmune disease may result.
- Induced tolerance. This is tolerance to external antigens that has been created by deliberately manipulating the immune system. Its importance:
  - to protect us from unpleasant, even dangerous, allergic reactions to such things as food (e.g. peanuts), insect stings, grass pollen (hay fever).
  - to enable transplanted organs (e.g., kidney, heart, liver) to survive in their new host; that is, to avoid graft rejection.
  - to reveal the mechanisms of autoimmunity in the hope of designing treatments for such diseases as systemic lupus erythematosus (SLE) and multiple sclerosis (MS).
- Immunological tolerance is not simply a failure to recognize an antigen; it is an active response to a particular epitope and is just as specific as an immune response.
- Both B cells and T cells can be made tolerant, but it is more important to tolerize T cells than B cells because B cells cannot make antibodies to most antigens without the help of T cells.

T-cell Tolerance

Central Tolerance

In the thymus, the epitopes recognized by these receptors consist of:

- a small molecule, usually a peptide of 6–8 amino acids derived from body proteins; that is, "self" proteins nestled in [View]
- a histocompatibility molecule (encoded by the MHC)
  - class II for $CD4^+$ T cells
  - class I for $CD8^+$ T cells

T cells whose receptors bind these epitopes so tightly that they could attack the cell displaying them are deleted by apoptosis. The T cells that survive this negative selection leave the thymus and migrate throughout the immune system (lymph nodes, spleen, etc.).

- The antigen-presenting cells in the thymus are certainly capable of presenting peptide fragments from the many "housekeeping" proteins found in all cells (e.g., the enzymes used in glycolysis). But there are many proteins that are expressed only in differentiated cells that are restricted to a particular tissue e.g., the insulin-producing beta cells in the islets of Langerhans in the pancreas. How is central tolerance to these proteins achieved in the thymus?
- It turns out that antigen-presenting cells in the medulla of the thymus express a gene, *AIRE*, that encodes a transcription factor that turns on the expression of hundreds of tissue-specific genes encoding such proteins as
  - the precursor to insulin
  - thyroglobulin (precursor of the thyroxine secreted by the thyroid gland)
  - casein (protein in the milk secreted by the mammary glands) a protein secreted by the salivary glands
- The AIRE protein does not seem to increase the expression of housekeeping genes. How it distinguishes between these and the tissue-specific genes to be turned on remains to be discovered.
- AIRE protein binds to chromatin whose histone H3 has no methyl groups attached to its lysine-4 ("H3K4me^0". This is a mark of inactive genes.
- AIRE stands for autoimmune regulator. Knockout mice and those rare humans who have no functioning *AIRE* gene suffer from severe autoimmune disease especially of their various endocrine organs.

Peripheral Tolerance

- The T cells that leave the thymus are relatively — but not completely — safe. Some will have receptors (TCRs) that can respond to self antigens
  - that are present in such high concentration that they can bind to "weak" receptors;
  - that they may not have encountered in the thymus.

Thanks to the activity of AIRE, the list of the latter molecules

Five possibilities for which there is substantial evidence:

1. Negative Selection in the Peripheral Immune System

AIRE is also active in some antigen-presenting cells in the organs of the peripheral immune system, e.g., lymph nodes and spleen. So any potentially autoreactive T cells that failed to be eliminated in the thymus can be selected against in these tissues.

2. Lack of Costimulation

The binding of a T cell to an antigen-presenting cell (APC) is by itself not enough to activate the T cell and turn it into an effector cell: one able to, for examples,

- kill the APC ($CD8^+$ cytotoxic T lymphocytes [CTLs])
- carry out cell-mediated immune reactions ($CD4^+$ Th1 cells)
- provide help to B cells ($CD4^+$ Th2 cells)
- may not be as large as we once thought.

Although T cells encounter self antigens in body tissues, they will not respond unless they receive a second signal. In fact, binding of their TCR ("signal one") without "signal two" causes them to self-destruct by apoptosis. Most of the time, the cells presenting the body's own antigens either

fail to provide signal two or transmit an as-yet-unidentified second signal that turns the T cell into a regulatory T cell (Treg) that suppresses immune responses.

In either case, self-tolerance results

3. Failure to Encounter Self Antigens

- Some tissues are hidden behind anatomical barriers that keep T cells from reaching them. Examples of such "privileged sites":
  - interior of the eye
  - testes
  - the brain
- Mechanical damage can breach the barrier and an autoimmune attack follow.

4. Receipt of Death Signals

- Some cells of the body express the Fas ligand, FasL. Activated T cells always express Fas. When they encounter these cells, binding of Fas to FasL triggers their death by apoptosis.

Examples:

- Cells within the eye always express FasL and are thus ready to kill off any rogue T cells that might gain entry.

Macrophages infected with HIV express FasL and thus kill any anti-HIV T cells that try to kill them. This may account for the disastrous decline in $CD4^+$ T cells late in the development of AIDS.

5. Control by Regulatory T Cells

- A minor population of $CD4^+$ T cells, called regulatory T cells, suppresses the activity of other T cells. They may be important players in protecting the body from attack by its other T cells.

B-cell Tolerance

- The problem of B-cell tolerance is not so acute because B cells cannot respond to most antigens unless they receive help from T helper cells.
- Nevertheless, B cells become tolerized to self components and, like T cells, this occurs both in the bone marrow (central tolerance) and elsewhere in the body (peripheral tolerance).

Central Tolerance

- B cells are formed and mature in the bone marrow. In humans, over half of the developing B cells produce a BCR able to bind self components.
- Any cells that produce a receptor for antigen (BCR) that would bind self components too tightly undergo a process of receptor editing. They dip again into their pool of gene segments that encode the light and heavy chains of their BCR and try to make a new BCR that is not a threat. If they fail, they commit suicide (apoptosis).
- Despite these mechanisms, some of the B cells that migrate out of the bone marrow continue to express self-reactive BCRs and may still be able to produce anti-self antibodies. So a mechanism is needed to tolerize them out in the tissues ("peripheral tolerance").

Peripheral Tolerance

- B cells with a potential for attacking self can be kept in check by the absence of the T-helper cells they need; that is, T-cell tolerance is probably the most important (but not the only) mechanism for maintaining B-cell tolerance.

Induced Tolerance

Allergies

Allergists have struggled for years to find safe ways to tolerize allergic people to their allergens. This has usually involved giving a long series of injections of a special formulation of the allergen.

Examples:

- the active ingredient in poison ivy that triggers this cell-mediated immune response;
- allergens that trigger IgE-mediated allergic responses, such as
  - ragweed, grass, and tree pollens;
  - insect stings;
  - food allergens, e.g., peanuts and other nuts

Transplant Tolerance

If ways could be found to induce genuine tolerance to allografts (organs transplanted from another person), this would enable the organ to resist rejection without the need for continuous use of immunosuppressive drugs.

It also has inspired attempts to achieve graft tolerance in humans by pretreating the recipient with blood (rich in B cells) or bone marrow of the donor.

In such cases (as well as Billingham's), it may be that tolerance of the graft is

- created because the priming cells are unable to give a second signal to host T cells and
- maintained by the continued survival in the recipient of these donor cells.

Tolerance of the Fetus

- The human fetus is also an allograft, but the mother makes no attempt to reject it (at least for 9 months). How this tolerance is established and maintained is still being studied.

IMMUNODEFICIENCY DISORDERS

- Types
- Risk factors
- Signs
- Diagnosis
- Treatment
- Prevention
- Outlook

Key points

1. Immunodeficiency disorders disrupt your body's ability to defend itself against bacteria, viruses, and parasites.
2. There are two types of immunodeficiency disorders: those you are born with (primary), and those that are acquired (secondary).
3. Anything that weakens your immune system can lead to a secondary immunodeficiency disorder.

Immunodeficiency disorders prevent your body from fighting infections and diseases. This type of disorder makes it easier for you to catch viruses and bacterial infections.

Immunodeficiency disorders are either congenital or acquired. A congenital, or primary, disorder is one you were born with. Acquired, or secondary, disorders you get later in life. Acquired disorders are more common than congenital disorders.

Your immune system includes the following organs:

- spleen
- tonsils
- bone marrow
- lymph nodes

These organs make and release lymphocytes. These are white blood cells classified as B cells and T cells. B and T cells fight invaders called antigens. B cells release antibodies specific to the disease your body detects. T cells destroy foreign or abnormal cells.

Examples of antigens that your B and T cells might need to fight off include:

- bacteria
- viruses
- cancer cells

- parasites

An immunodeficiency disorder disrupts your body's ability to defend itself against these antigens.

What are the different types of immunodeficiency disorders?

An immune deficiency disease occurs when the immune system is not working properly.

- primary (congenital) immunodeficiency that result of: – Defects in B and T lymphocytes maturation – Defects in lymphocytes activation and function – Defects in innate immunity – Lymphocyte abnormalities associated with other diseases
- acquired (secondary) immunodeficiency syndrome (AIDS) in regard of: – Structure and biology of HIV – Clinical features of HIV infection and pathogenesis of AIDS – AIDS therapeutic and vaccination strategies

If you are born with a deficiency or if there is a genetic cause, it is called primary immunodeficiency disease. There are more than 100 primary immunodeficiency disorders.

Examples of primary immunodeficiency disorders include:

- X-linked agammaglobulinemia (XLA)
- common variable immunodeficiency (CVID)
- severe combined immunodeficiency (SCID), which is known as alymphocytosis or "boy in a bubble" disease

Secondary immunodeficiency disorders happen when an outside source like a toxic chemical or infection attacks your body. The following can cause a secondary immunodeficiency disorder:

- severe burns
- chemotherapy
- radiation
- diabetes
- malnutrition

Examples of secondary immunodeficiency disorders include:

- AIDS
- cancers of the immune system, like leukemia
- immune-complex diseases, like viral hepatitis
- multiple myeloma (cancer of the plasma cells, which produce antibodies)

Type of immunodeficiency	Histopathology and laboratory abnormalities	Common infectious consequences
B cell deficiencies	Absent or reduced follicles and germinal centers in lymphoid organs Reduced serum Ig levels	Pyogenic bacterial infections
T cell deficiencies	May be reduced T cell zones in lymphoid organs Reduced DTH reactions to common antigens Defective T cell proliferative responses to mitogens *in vitro*	Viral and other intracellular microbial infections (e.g., *Pneumocystis carinii*, atypical mycobacteria, fungi) Virus-associated malignancies (e.g., EBV-associated lymphomas)
Innate immune deficiencies	Variable, depending on which component of innate immunity is defective	Variable; pyogenic bacterial infections

Congenital Immunodeficiencies

- **Primary Immunodeficiencies are congenital diseases caused by genetic defects that inhibitmaturation of B or T cells or interfere with functions of immune components**
- **Prevalence**: 1 in 500 American and European
- **Hallmark of immunodeficiencies' consequences:**
  - ***Infections that could be mild or severe, start in earlychildhood or in adulthood***

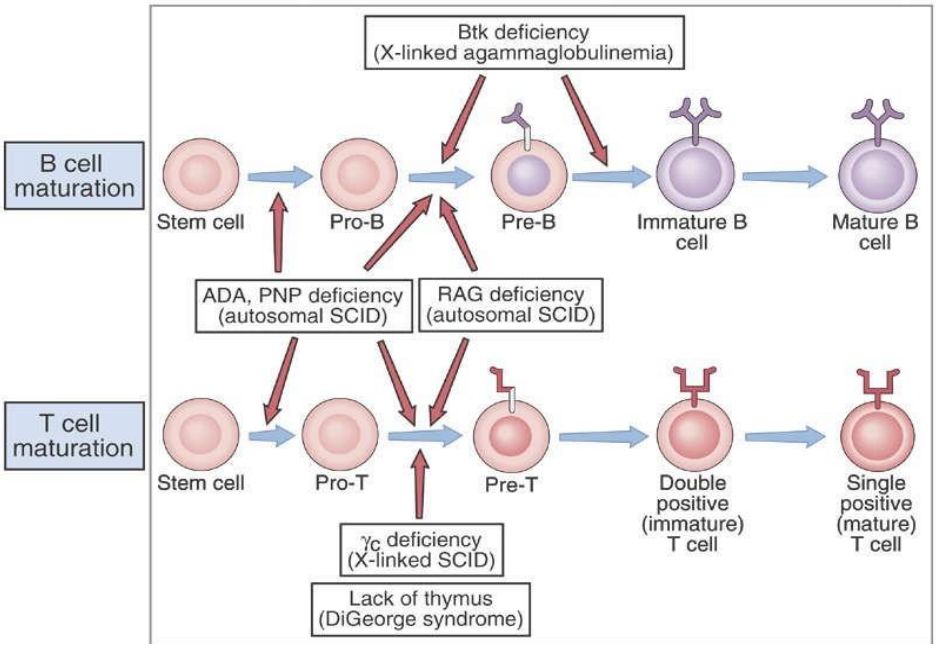
Congenital Immunodeficies caused by defecs in Lymphocyte maturation

- Autosomal SCID (severe combined immunodeficiency) caused by:
  - **ADA** (adenosine deaminase) & **PNP** (purine nucleosidephosphorylase) **deficiency** prevent maturation of:
    - Stem cell → **pro-B cell**
    - Pro-B cell → **pre-B cell**
    - Stem cell →**pro-T cell**
    - Pro-T cell → **pre-T cell**

- **RAG** (recombination activating gene) deficiency preventsmaturation of:
  - Pro-B cell → pre-B cell
  - Pro-T cell → pre-T cell

- ***X-linked SCID:***
  - **Signaling IL-2Rg chain** (□c is common in IL receptors of IL-2,IL-4, **IL-7**, IL-9, IL-15) deficiency interferes with

    Pro-T cell → pre-T cell



Features of congenital immunodeficiencies caused by defects in lymphocyte maturation

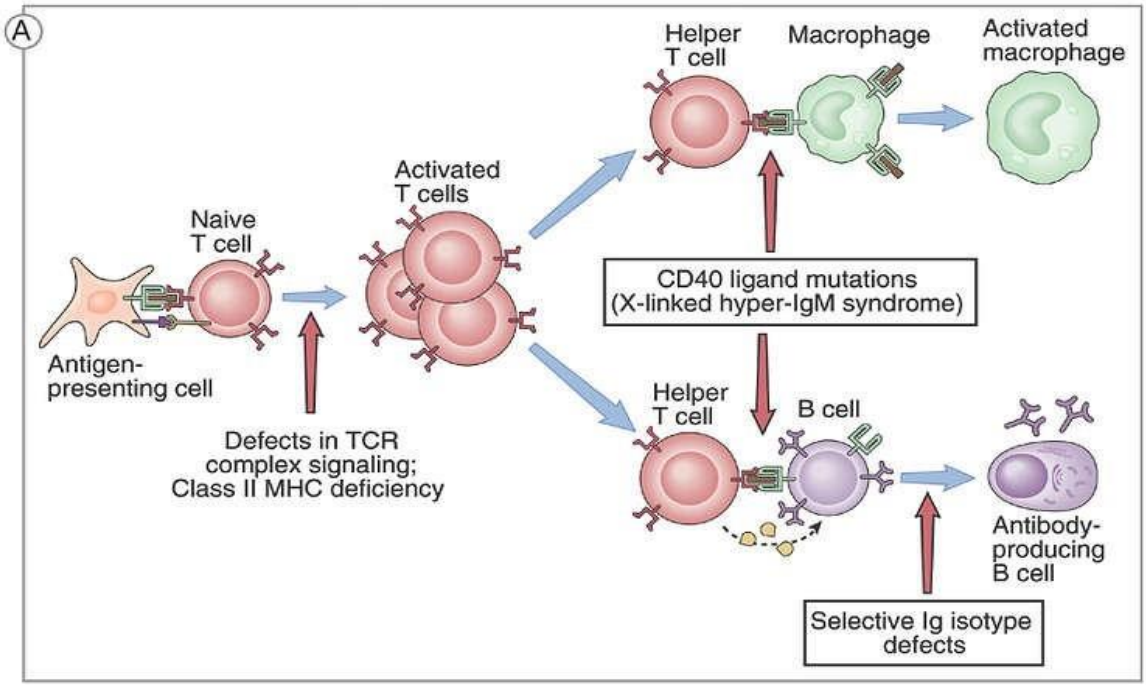
Severe combined immunodeficiency (SCID)		
Disease	Functional deficiencies	Mechanism of defect
X-linked SCID	Markedly decreased T cells; normal or increased B cells; reduced serum Ig	Cytokine receptor common γ chain gene mutations, defective T cell maturation due to lack of IL-7 signals
Autosomal recessive SCID due to ADA, PNP deficiency	Progressive decrease in T and B cells (mostly T); reduced serum Ig in ADA deficiency, normal B cells and serum Ig in PNP deficiency	ADA or PNP deficiency leads to accumulation of toxic metabolites in lymphocytes
Autosomal recessive SCID due to other causes	Decreased T and B cells; reduced serum Ig	Defective maturation of T and B cells; genetic basis unknown in most cases; may be mutations in *RAG* genes

Features of congenital immunodeficiencies caused by defects in lymphocyte maturation

B cell immunodeficiencies		
Disease	Functional deficiencies	Mechanism of defect
X-linked agammaglobulinemia	Decrease in all serum Ig isotypes; reduced B cell numbers	Block in maturation beyond pre-B cells, because of mutation in B cell tyrosine kinase
Ig heavy chain deletions	IgG1, IgG2, or IgG4 absent; sometimes associated with absent IgA or IgE	Chromosomal deletion at 14q32 (Ig heavy chain locus)

T cell immunodeficiencies		
Disease	Functional deficiencies	Mechanism of defect
DiGeorge syndrome	Decreased T cells; normal B cells; normal or decreased serum Ig	Anomalous development of 3rd and 4th branchial pouches, leading to thymic hypoplasia

Features of congenital immunodeficiencies caused by defects in lymphocyte activation and effector functions



Congenital immunodeficiencies may be caused by genetic defects in the expression of molecules required for

– Ag presentation to T cells,

– T or B lymphocyte antigen receptor signaling,

– helper T cell activation of B cells and macrophages, and

– differentiation of antibody-producing B cells.

Ⓑ Disease	Functional Deficiencies	Mechanisms of Defect
X-linked hyper-IgM syndrome	Defects in helper T cell–dependent B cell and macrophage activation	Mutations in CD40 ligand
Selective immunoglobulin isotype deficiencies	Reduced or no production of selective isotypes or subtypes of immunoglobulins; susceptibility to bacterial infections or no clinical problems	Unknown; may be defect in B cell differentiation or T cell help
Defective class II MHC expression: The bare lymphocyte syndrome	Lack of class II MHC expression and impaired $CD4^+$ T cell activation; defective cell-mediated immunity and T cell–dependent humoral immunity	Mutations in genes encoding transcription factors required for class II MHC gene expression
Defects in T cell receptor complex expression or signaling	Decreased T cells or abnormal ratios of $CD4^+$ and $CD8^+$ subsets; decreased cell-mediated immunity	Rare cases due to mutations or deletions in genes encoding CD3 proteins, ZAP-70

Congenital immunodeficiencies caused by defects in innate immunity

Disease	Functional Deficiencies	Mechanisms of Defect
Chronic granulomatous disease	Defective production of reactive oxygen intermediates by phagocytes	Mutations in genes encoding components of the phagocyte oxidase enzyme, most often cytochrome b558
Leukocyte adhesion deficiency-1	Absent or deficient expression of β2 integrins causing defective leukocyte adhesion-dependent functions	Mutations in gene encoding the β chain (CD18) of β2 integrins
Leukocyte adhesion deficiency-2	Absent or deficient expression of leukocyte ligands for endothelial E- and P-selectins, causing failure of leukocyte migration into tissues	Mutations in gene encoding a protein required for synthesis of the sialyl-Lewis X component of E- and P-selectin ligands

Disease	Functional Deficiencies	Mechanisms of Defect
Complement C3 deficiency	Defect in complement cascade activation	Mutations in the C3 gene
Complement C2, C4 deficiency	Deficient activation of classical pathway of complement leading to failure to clear immune complexes and development of lupus-like disease	Mutations in C2 or C4 genes
Chédiak-Higashi syndrome	Defective lysosomal function in neutrophils, macrophages and dendritic cells, and defective granule function in natural killer cells	Mutation in a gene encoding a lysosomal trafficking regulatory protein

Acquired (secondary) immunodeficiency diseases

Cause	Mechanism
Human immunodeficiency virus infection	Depletion of $CD4^+$ helper T cells
Protein-calorie malnutrition	Metabolic derangements inhibit lymphocyte maturation and function
Irradiation and chemotherapy treatments for cancer	Decreased bone marrow precursors for all leukocytes
Cancer metastases to bone marrow	Reduced site of leukocyte development
Removal of spleen	Decreased phagocytosis of microbes

TRANSPLANTATION IMMUNOLOGY

Introduction

Today, kidney, pancreas, heart, lung, liver, bone marrow, and cornea transplantations are performed among non-identical individuals with ever increasing frequency and success

Classification

- **Autologous grafts (Autografts)**
  - Grafts transplanted from one part of the body to another in the same individual
- **Syngeneic grafts (Isografts)**
  - Grafts transplanted between two genetically identical individuals of the same species
- **Allogeneic grafts (Allografts)**
  - Grafts transplanted between two genetically different individuals of the same species
- **Xenogeneic grafts (Xenografts)**
  - Grafts transplanted between individuals of different species

Graft Rejection

- Grafts rejection is a kind of specific immune response
  - Specificity
  - Immune memory
- Grafts rejection
  - First set rejection
  - Second set rejection

Immunologic Basis of Allograft Rejection

I. Transplantation antigens

- Major histocompatibility antigens (MHC molecules)
- Minor histocompatibility antigens
- Other alloantigens

1. Major histocompatibility antigens
   a. Main antigens of grafts rejection
   b. Cause fast and strong rejection
   c. Difference of HLA types is the main cause of human grafts rejection
2. Minor histocompatibility antigens
   a. Also cause grafts rejection, but slow and weak
   b. Mouse H-Y antigens encoded by Y chromosome
   c. HA-1～HA-5 linked with non-Y chromosome

3. Other alloantigens

a. Human ABO blood group antigens

b. Some tissue specific antigens

i. Skin > kidney > heart > pancreas > liver

ii. VEC antigen

iii. SK antigen

II. Mechanism of allograft rejection

1. Cell-mediated Immunity

Recipient's T cell-mediated cellular immune response against alloantigens on grafts

Molecular Mechanisms of Allogeneic Recognition:

? T cells of the recipient recognize the allogenetic MHC molecules

? Many T cells can recognize allogenetic MHC molecules

- 10^{-5}-10^{-4} of specific T cells recognize conventional antigens
- 1%-10% of T cells recognize allogenetic MHC molecules

? The recipient' T cells recognize the allogenetic MHC molecules

➢ Direct Recognition

- Recognition of an intact allogenetic MHC molecule displayed by donor APC in the graft
- Cross recognition

– An allogenetic MHC molecule with a bound peptide can mimic the determinant formed by a self MHC molecule plus foreign peptide

– A cross-reaction of a normal TCR, which was selected to recognize a self MHC molecules plus foreign peptide, with an allogenetic MHC molecule plus peptide

- Passenger leukocytes

– Donor APCs that exist in grafts, such as DC, MΦ

– Early phase of acute rejection ?

– Fast and strong ?

? Many T cells can recognize allogenetic MHC molecules

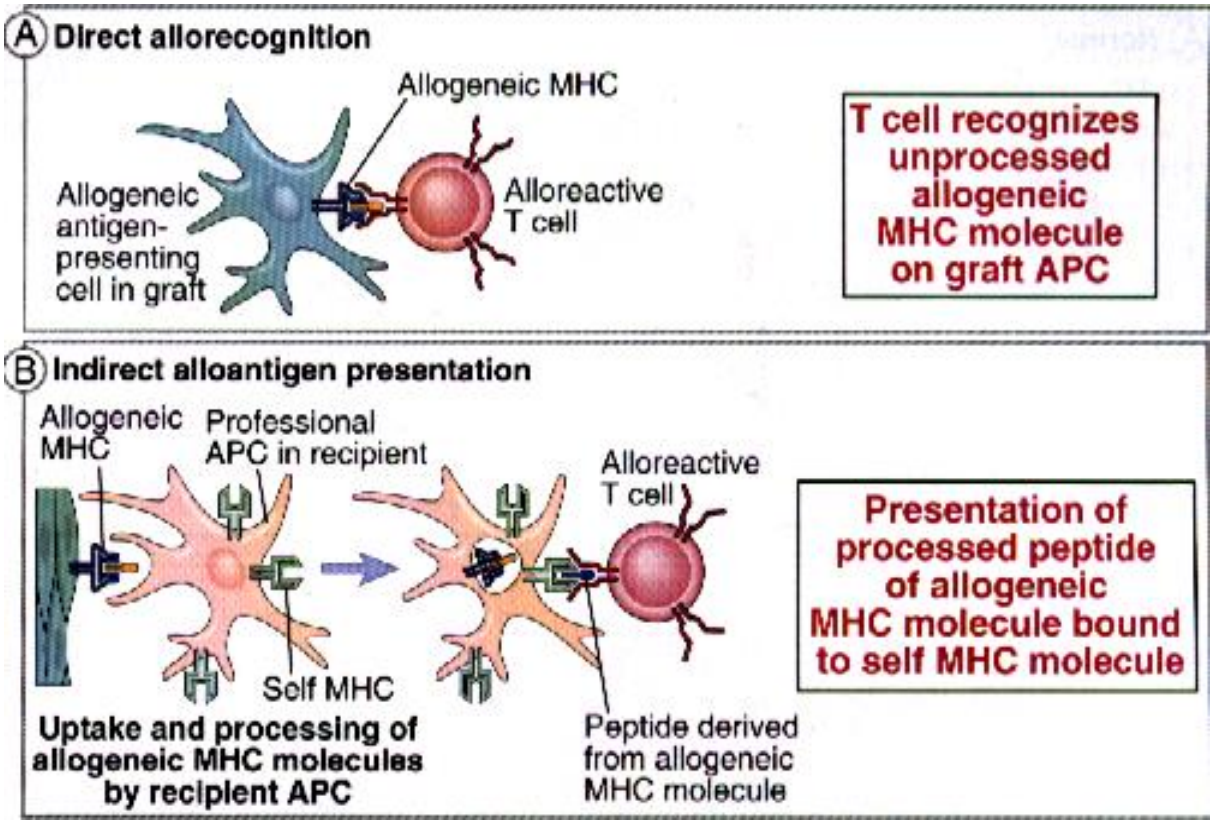
– Allogenetic MHC molecules (different residues)

– Allogenetic MHC molecules–different peptides

- All allogenetic MHC molecules on donor APC can be epitopes recognized by TCR

➢ Indirect Recognition

- Uptake and presentation of allogeneic donor MHC molecules by recipient APC in "normal way"
- Recognition by T cells like conventional foreign antigens



Difference between Direct Recognition and Indirect Recognition

	Direct Recognition	**Indirect Recognition**
Allogeneic MHC molecule	Intact allogeneic MHC molecule	Peptide of allogeneic MHC molecule
APCs	Recipient APCs are not necessary	Recipient APCs

Activated T cells	$CD4^+$T cells and/or $CD8^+$T cells	$CD4^+$T cells and/or $CD8^+$T cells
Roles in rejection	Acute rejection	Chronic rejection
Degree of rejection	Vigorous	Weak

Role of $CD4^+$T cells and $CD8^+$T cells

- Activated $CD4^+$T by direct and indirect recognition
  - CK secretion
  - MΦ activation and recruitment
- Activated $CD8^+$T by direct recognition
  - Kill the graft cells directly
- Activated $CD8^+$T by indirect recognition
  - Can not kill the graft cells directly

2. Humoral Immunity

- Important role in hyperacute rejection

(Preformed antibodies)

  - Complements activation
  - ADCC
  - Opsonization
- Enhancing antibodies

/Blocking antibodies

3. Role of NK cells

- KIR can't recognize allogeneic MHC on graft
- CKs secreted by activated Th cells can promote NK activation

Classification and Effector Mechanisms of Allograft Rejection

Classification of Allograft Rejection

- Host versus graft reaction (HVGR)
  - Conventional organ transplantation
- Graft versus host reaction (GVHR)

- Bone marrow transplantation
- Immune cells transplantation

I. Host versus graft reaction (HVGR)

a. **Hyperacute rejection**

i. Occurrence time

- Occurs within minutes to hours after host blood vessels are anastomosed to graft vessels

ii. Pathology

- Thrombotic occlusion of the graft vasculature
- Ischemia, denaturation, necrosis

iii. Mechanisms

- Preformed antibodies

a. Antibody against ABO blood type antigen

b. Antibody against VEC (Vascular Endothelial Cell) antigen

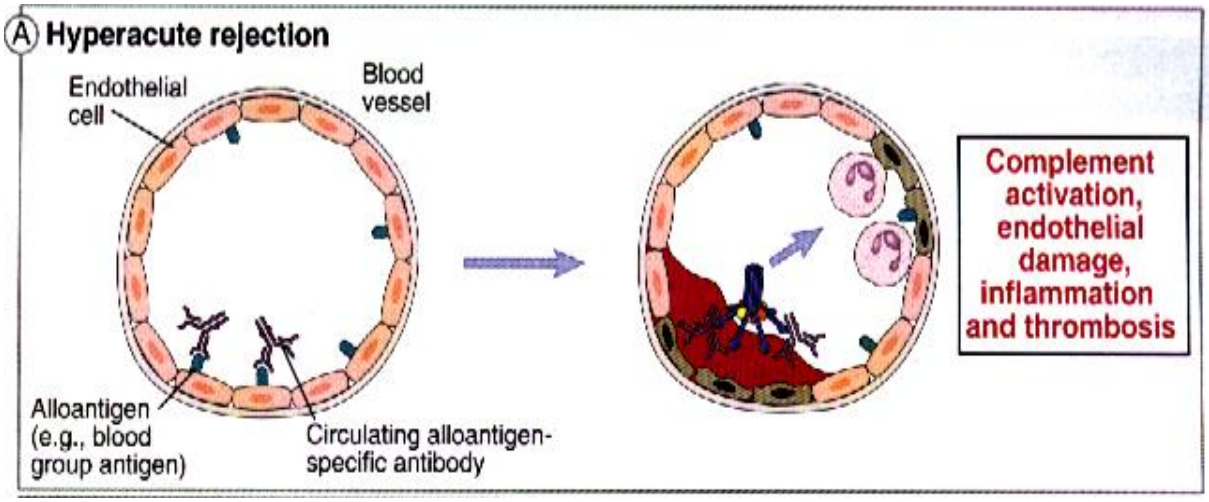
c. Antibody against HLA antigen

- Complement activation

a. Endothelial cell damage

- Platelets activation

a. Thrombosis, vascular occlusion, ischemic damage



b. **Acute rejection**

i. Occurrence time

- Occurs within days to 2 weeks after transplantation, 80-90% of cases occur within 1 month

ii. Pathology

- Acute humoral rejection

a. Acute vasculitis manifested mainly by endothelial cell damage

– Acute cellular rejection

a. Parenchymal cell necrosis along with infiltration of lymphocytes and MΦ

iii. Mechanisms

– Vasculitis

a. IgG antibodies against alloantigens on endothelial cell

b. CDC

– Parench ymal cell damage

a. Delayed hypersensitivity mediated by CD4+Th1

b. Killing of graft cells by CD8+Tc

c. **Chronic rejection**

i. Occurrence time

– Develops months or years after acute rejection reactions have subsided

ii. Pathology

– Fibrosis and vascular abnormalities with loss of graft function

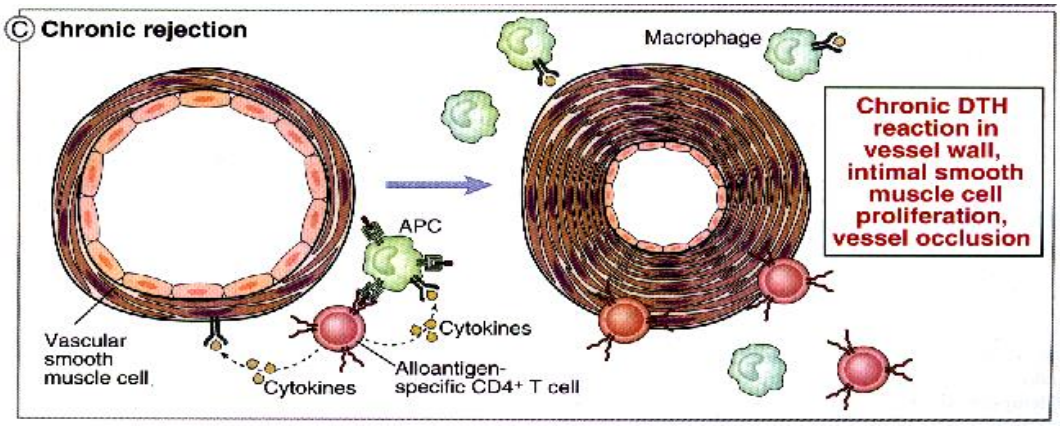
iii. Mechanisms

– Not clear

– Extension and results of cell necrosis in acute rejection

– Chronic inflammation mediated by CD4+T cell/MΦ

– Organ degeneration induced by non immune factors



II. Graft versus host reaction (GVHR)

a. Graft versus host reaction (GVHR)

i. Allogenetic bone marrow transplantation

ii. Rejection to host alloantigens

iii. Mediated by immune competent cells in bone marrow

b. Graft versus host disease (GVHD)

i. A disease caused by GVHR, which can damage the host

1. Acute GVHD

a. Endothelial cell death in the skin, liver, and gastrointestinal tract

b. Rash, jaundice, diarrhea, gastrointestinal hemorrhage

c. Mediated by mature T cells in the grafts

2. Chronic GVHD

a. Fibrosis and atrophy of one or more of the organs

b. Eventually complete dysfunction of the affected organ

Both acute and chronic GVHD are commonly treated with intense immunosuppresion

- Uncertain
- Fatal

Prevention and Therapy of Allograft Rejection

- **Tissue Typing**
- ABO and Rh blood typing
- Crossmatching (Preformed antibodies)
- HLA typing
  - HLA-A and HLA-B
  - HLA-DR
-
- **Immunosuppressive Therapy**
- Cyclosporine(CsA), FK506
  - Inhibit NFAT transcription factor
- Azathioprine, Cyclophosphamide
  - Block the proliferation of lymphocytes
- Ab against T cell surface molecules
  - Anti-CD3 mAb----Deplete T cells
- Anti-inflammatory agents
  - Corticosteroids----Block the synthesis and secretion of cytokines
- **Induction of Immune Tolerance**

Immunosuppression

Immunosuppression involves an act that reduces the activation or efficacy of the immune system. Some portions of the immune system itself have immuno-suppressive effects on other parts of the immune system, and immunosuppression may occur as an adverse reaction to treatment of other conditions.

In general, deliberately induced immunosuppression is performed to prevent the body from rejecting an organ transplant, treating graft-versus-host disease after a bone marrow transplant, or for the treatment of auto-immune diseases such as rheumatoid arthritis or Crohn's disease. This is typically done using drugs, but may involve surgery (splenectomy), plasmapharesis, or radiation.

A person who is undergoing immunosuppression, or whose immune system is weak for other reasons (for example, chemotherapy and HIV), is said to be *immunocompromised*. An **immunosuppressant** is any agent that causes immunosuppression, including immunosuppressive drugs and some environmental toxins.

Deliberately induced

Administration of immunosuppressive drugs or immunosuppressants is the main method of deliberately induced immunosuppression. In optimal circumstances, immunosuppressive drugs are targeted only at any hyperactive component of the immune system, and in ideal circumstances would not cause any significant immunodeficiency. However, in essence, all immunosuppressive drugs have the potential to cause immunodeficiency. Immunodeficiency may manifest as increased susceptibility to opportunistic infections and decreased cancer immunosurveillance. The term *immunotoxin* is also sometimes used (incorrectly) to label undesirable immunosuppressants, such as various pollutants. Polychlorinated biphenyls (PCB) and the insecticide DDT are immunosuppressants. Immunosuppressants may be prescribed when a normal immune response is undesirable, such as in autoimmune diseases.

Cortisone was the first immunosuppressant identified, but its wide range of side-effects limited its use. The more specific azathioprine was identified in 1959, but it was the discovery of cyclosporine in 1970 that allowed for significant expansion of kidney transplantation to less well-matched donor-recipient pairs as well as broad application of liver transplantation, lung transplantation, pancreas transplantation, and heart transplantation. After an organ transplantation, the body will nearly always reject the new organ(s) due to differences in human leukocyte antigen haplotypes between the donor and recipient. As a result, the immune system detects the new tissue as "hostile", and attempts to remove it by attacking it with recipient leukocytes, resulting in the death of the tissue. Immunosuppressants are applied as a

countermeasure; the side-effect is that the body becomes more vulnerable to infections and malignancy, much like in an advanced HIV infection.

Throughout its history, radiation therapy has been used to decrease the strength of the immune system. Dr. Joseph Murray of Harvard Medical School and chief plastic surgeon at Children's Hospital Boston from 1972-1985 were awarded the Nobel Prize in Physiology or Medicine in 1990 for his work on immunosuppression.

Non-deliberate immunosuppression

Non-deliberate immunosuppression can occur in, for example, malnutrition, aging, many types of cancer (such as leukemia, lymphoma, multiple myeloma), and certain chronic infections such as Human Immunodeficiency virus (HIV). The unwanted effect in non-deliberate immunosuppression is immunodeficiency that results in increased susceptibility to pathogens such as bacteria and virus.

Immunodeficiency is also a potential adverse effects of many immunosuppressant drugs. In this sense, the scope of the term *immunosuppression* in general includes both beneficial and potential adverse effects of decreasing the function of the immune system, whereas the term *immunodeficiency* in general refers solely to the adverse effect of increased risk for infection.

Immunosuppressive drug

Immunosuppressive drugs or **immunosuppressive agents** or **antirejection medications** are drugs that inhibit or prevent activity of the immune system. They are used in immunosuppressive therapy to:

- Prevent the rejection of transplanted organs and tissues (e.g., bone marrow, heart, kidney, liver)
- Treat autoimmune diseases or diseases that are most likely of autoimmune origin (e.g., rheumatoid arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, sarcoidosis, focal segmental glomerulosclerosis, Crohn's disease, Behcet's Disease, pemphigus, and ulcerative colitis).
- Treat some other non-autoimmune inflammatory diseases (e.g., long term allergic asthma control).

A common side-effect of many immunosuppressive drugs is immunodeficiency, because the majority of them act non-selectively, resulting in increased susceptibility to infections and decreased cancer immunosurveillance. There are also other side-effects, such as hypertension, dyslipidemia, hyperglycemia, peptic ulcers, lipodystrophy, moon face, liver and kidney injury. The immunosuppressive drugs also interact with other medicines and affect their metabolism

and action. Actual or suspected immunosuppressive agents can be evaluated in terms of their effects on lymphocyte subpopulations in tissues using immunohistochemistry.

Immunosuppressive drugs can be classified into five groups:

- glucocorticoids
- cytostatics
- antibodies
- drugs acting on immunophilins
- other drugs.

1. Glucocorticoids:

In pharmacologic (supraphysiologic) doses, glucocorticoids are used to suppress various allergic, inflammatory, and autoimmune disorders. They are also administered as posttransplantory immunosuppressants to prevent the acute transplant rejection and graft-versus-host disease. Nevertheless, they do not prevent an infection and also inhibit later reparative processes.

1.1. Immunosuppressive mechanism

Glucocorticoids suppress the cell-mediated immunity. They act by inhibiting genes that code for the cytokines Interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and TNF-γ, the most important of which is IL-2. Smaller cytokine production reduces the T cell proliferation.

Glucocorticoids also suppress the humoral immunity, causing B cells to express smaller amounts of IL-2 and IL-2 receptors. This diminishes both B cell clone expansion and antibody synthesis.

1.2. Anti-inflammatory effects

Glucocorticoids influence all types of inflammatory events, no matter their cause. They induce the lipocortin-1 (annexin-1) synthesis, which then binds to cell membranes preventing the phospholipase A2 from coming into contact with its substrate arachidonic acid. This leads to diminished eicosanoid production. The cyclooxygenase (both COX-1 and COX-2) expression is also suppressed, potentiating the effect.

Glucocorticoids also stimulate the lipocortin-1 escaping to the extracellular space, where it binds to the leukocyte membrane receptors and inhibits various inflammatory events: epithelial adhesion, emigration, chemotaxis, phagocytosis, respiratory burst, and the release of various inflammatory mediators (lysosomal enzymes, cytokines, tissue plasminogen activator, chemokines, etc.) from neutrophils, macrophages, and mastocytes.

2. Cytostatics:

Cytostatics inhibit cell division. In immunotherapy, they are used in smaller doses than in the treatment of malignant diseases. They affect the proliferation of both T cells and B cells. Due to their highest effectiveness, purine analogs are most frequently administered.

2.1. Alkylating agents

The alkylating agents used in immunotherapy are nitrogen mustards (cyclophosphamide), nitrosoureas, platinum compounds, and others. Cyclophosphamide (Baxter's Cytoxan) is probably the most potent immunosuppressive compound. In small doses, it is very efficient in the therapy of systemic lupus erythematosus, autoimmune hemolytic anemias, Wegener's granulomatosis, and other immune diseases. High doses cause pancytopenia and hemorrhagic cystitis.

2.2. Antimetabolites

Antimetabolites interfere with the synthesis of nucleic acids. These include:

- folic acid analogues, such as methotrexate
- purine analogues, such as azathioprine and mercaptopurine
- pyrimidine analogues, such as fluorouracil
- protein synthesis inhibitors.

Methotrexate

Methotrexate is a folic acid analogue. It binds dihydrofolate reductase and prevents synthesis of tetrahydrofolate. It is used in the treatment of autoimmune diseases (for example rheumatoid arthritis or Behcet's Disease) and in transplantations.

Azathioprine and mercaptopurine

Azathioprine (Prometheus' Imuran), is the main immunosuppressive cytotoxic substance. It is extensively used to control transplant rejection reactions. It is nonenzymatically cleaved to mercaptopurine, that acts as a purine analogue and an inhibitor of DNA synthesis. Mercaptopurine itself can also be administered directly.

By preventing the clonal expansion of lymphocytes in the induction phase of the immune response, it affects both the cell and the humoral immunity. It is also efficient in the treatment of autoimmune diseases.

Cytotoxic antibiotics

Among these, dactinomycin is the most important. It is used in kidney transplantations. Other cytotoxic antibiotics are anthracyclines, mitomycin C, bleomycin, mithramycin.

3. Antibodies:

Antibodies are sometimes used as a quick and potent immunosuppressive therapy to prevent the acute rejection reactions as well as a targeted treatment of lymphoproliferative or autoimmune disorders (e.g., anti-CD20 monoclonals).

3.1. Polyclonal antibodies

Heterologous polyclonal antibodies are obtained from the serum of animals (e.g., rabbit, horse), and injected with the patient's thymocytes or lymphocytes. The antilymphocyte (ALG) and antithymocyte antigens (ATG) are being used. They are part of the steroid-resistant acute rejection reaction and grave aplastic anemia treatment. However, they are added primarily to other immunosuppressives to diminish their dosage and toxicity. They also allow transition to ciclosporin therapy.

Polyclonal antibodies inhibit T lymphocytes and cause their lysis, which is both complement-mediated cytolysis and cell-mediated opsonization followed by removal of reticuloendothelial cells from the circulation in the spleen and liver. In this way, polyclonal antibodies inhibit cell-mediated immune reactions, including graft rejection, delayed hypersensitivity (i.e., tuberculin skin reaction), and the graft-versus-host disease (GVHD), but influence thymus-dependent antibody production.

As of March 2005, there are two preparations available to the market: Atgam, obtained from horse serum, and Thymoglobuline, obtained from rabbit serum. Polyclonal antibodies affect all lymphocytes and cause general immunosuppression, possibly leading to post-transplant lymphoproliferative disorders (PTLD) or serious infections, especially by cytomegalovirus. To reduce these risks, treatment is provided in a hospital, where adequate isolation from infection is available. They are usually administered for five days intravenously in the appropriate quantity. Patients stay in the hospital as long as three weeks to give the immune system time to recover to a point where there is no longer a risk of serum sickness.

Because of a high immunogenicity of polyclonal antibodies, almost all patients have an acute reaction to the treatment. It is characterized by fever, rigor episodes, and even anaphylaxis. Later during the treatment, some patients develop serum sickness or immune complex glomerulonephritis. Serum sickness arises seven to fourteen days after the therapy has begun. The patient suffers from fever, joint pain, and erythema that can be soothed with the use of steroids and analgesics. Urticaria (hives) can also be present. It is possible to diminish their toxicity by using highly purified serum fractions and intravenous administration in the combination with other immunosuppressants, for example, calcineurin inhibitors, cytostatics and cortisteroids. The most frequent combination is to use antibodies and ciclosporin

simultaneously in order to prevent patients from gradually developing a strong immune response to these drugs, reducing or eliminating their effectiveness.

3.2. Monoclonal antibodies

Monoclonal antibodies are directed towards exactly defined antigens. Therefore, they cause fewer side-effects. Especially significant are the IL-2 receptor- (CD25-) and CD3-directed antibodies. They are used to prevent the rejection of transplanted organs, but also to track changes in the lymphocyte subpopulations. It is reasonable to expect similar new drugs in the future.

T-cell receptor directed antibodies

Muromonab-CD3 is a murine anti-CD3 monoclonal antibody of the IgG2a type that prevents T-cell activation and proliferation by binding the T-cell receptor complex present on all differentiated T cells. As such it is one of the most potent immunosuppressive substances and is administered to control the steroid- and/or polyclonal antibodies-resistant acute rejection episodes. As it acts more specifically than polyclonal antibodies it is also used prophylactically in transplantations.

The muromonab's mechanism of action is only partially understood. It is known that the molecule binds TCR/CD3 receptor complex. In the first few administrations this binding non-specifically activates T-cells, leading to a serious syndrome 30 to 60 minutes later. It is characterized by fever, myalgia, headache, and arthralgia. Sometimes it develops in a life-threatening reaction of the cardiovascular system and the central nervous system, requiring a lengthy therapy. Past this period CD3 blocks the TCR-antigen binding and causes conformational change or the removal of the entire TCR3/CD3 complex from the T-cell surface. This lowers the number of available T-cells, perhaps by sensitizing them for the uptake by the epithelial reticular cells. The cross-binding of CD3 molecules as well activates an intracellular signal causing the T cell anergy or apoptosis, unless the cells receive another signal through a co-stimulatory molecule. CD3 antibodies shift the balance from Th1 to Th2 cells.[*citation needed*]

The patient may develop neutralizing antibodies reducing the effectiveness of muromonab-CD3. Muromonab-CD3 can cause excessive immunosuppression. Although CD3 antibodies act more specifically than polyclonal antibodies, they lower the cell-mediated immunity significantly, predisposing the patient to opportunistic infections and malignancies.[*citation needed*]

IL-2 receptor directed antibodies

Interleukin-2 is an important immune system regulator necessary for the clone expansion and survival of activated lymphocytes T. Its effects are mediated by the trimer cell surface receptor IL-2a, consisting of the α, β, and γ chains. The IL-2a (CD25, T-cell activation antigen,

TAC) is expressed only by the already-activated T lymphocytes. Therefore, it is of special significance to the selective immunosuppressive treatment, and research has been focused on the development of effective and safe anti-IL-2 antibodies. By the use of recombinant gene technology, the mouse anti-Tac antibodies have been modified, leading to the presentation of two chimeric mouse/human anti-Tac antibodies in the year 1998: basiliximab (Simulect) and daclizumab (Zenapax). These drugs act by binding the IL-2a receptor's α chain, preventing the IL-2 induced clonal expansion of activated lymphocytes and shortening their survival. They are used in the prophylaxis of the acute organ rejection after bilateral kidney transplantation, both being similarly effective and with only few side-effects.

4. Drugs acting on immunophilins:

4.1. Ciclosporin

Like tacrolimus, ciclosporin (Novartis' Sandimmune) is a calcineurin inhibitor (CNI). It has been in use since 1983 and is one of the most widely used immunosuppressive drugs. It is a cyclic fungal peptide, composed of 11 amino acids.

Ciclosporin is thought to bind to the cytosolic protein cyclophilin (an immunophilin) of immunocompetent lymphocytes, especially T-lymphocytes. This complex of ciclosporin and cyclophilin inhibits the phosphatase calcineurin, which under normal circumstances induces the transcription of interleukin-2. The drug also inhibits lymphokine production and interleukin release, leading to a reduced function of effector T-cells.

Ciclosporin is used in the treatment of acute rejection reactions, but has been increasingly substituted with newer, and less nephrotoxic, immunosuppressants.

Calcineurin inhibitors and azathioprine have been linked with post-transplant malignancies and skin cancers in organ transplant recipients. Non-melanoma skin cancer (NMSC) after kidney transplantation is common and can result in significant morbidity and mortality. The results of several studies suggest that calcineurin inhibitors have oncogenic properties mainly linked to the production of cytokines that promote tumor growth, metastasis and angiogenesis.

This drug has been reported to reduce the frequency of regulatory T cells (T-Reg) and after converting from a CNI monotherapy to a mycophenolate monotherapy, patients were found to have increased graft success and T-Reg frequency.

4.2. Tacrolimus

Tacrolimus (trade name Prograf) is a product of the bacterium *Streptomyces tsukubaensis*. It is a macrolide lactone and acts by inhibiting calcineurin.

The drug is used primarily in liver and kidney transplantations, although in some clinics it is used in heart, lung, and heart/lung transplantations. It binds to the immunophilin FKBP1A,

followed by the binding of the complex to calcineurin and the inhibition of its phosphatase activity. In this way, it prevents the cell from transitioning from the G_0 into G_1 phase of the cell cycle. Tacrolimus is more potent than ciclosporin and has less pronounced side-effects.

4.3. Sirolimus

Sirolimus (rapamycin, trade name Rapamune) is a macrolide lactone, produced by the actinomycete bacterium *Streptomyces hygroscopicus*. It is used to prevent rejection reactions. Although it is a structural analogue of tacrolimus, it acts somewhat differently and has different side-effects.

Contrary to ciclosporin and tacrolimus, drugs that affect the first phase of T lymphocyte activation, sirolimus affects the second phase, namely signal transduction and lymphocyte clonal proliferation. It binds to FKBP1A like tacrolimus, however the complex does not inhibit calcineurin but another protein, mTOR. Therefore, sirolimus acts synergistically with ciclosporin and, in combination with other immunosuppressants, has few side effects. Also, it indirectly inhibits several T lymphocyte-specific kinases and phosphatases, hence preventing their transition from G_1 to S phase of the cell cycle. In a similar manner, Sirolimus prevents B cell differentiation into plasma cells, reducing production of IgM, IgG, and IgA antibodies.

It is also active against tumors that are PI3K/AKT/mTOR-dependent.

5. Other drugs:

5.1. Interferons

Main article: Interferon

IFN-β suppresses the production of Th1 cytokines and the activation of monocytes. It is used to slow down the progression of multiple sclerosis. IFN-γ is able to trigger lymphocytic apoptosis.

5.2. Opioids

Prolonged use of opioids may cause immunosuppression of both innate and adaptive immunity.[4] Decrease in proliferation as well as immune function has been observed in macrophages, as well as lymphocytes. It is thought that these effects are mediated by opioid receptors expressed on the surface of these immune cells.[4]

5.3. TNF binding proteins

A TNF-α (tumor necrosis factor-alpha) binding protein is a monoclonal antibody or a circulating receptor such as infliximab (Remicade), etanercept (Enbrel), or adalimumab (Humira) that binds to TNF-α, preventing it from inducing the synthesis of IL-1 and IL-6 and the adhesion of lymphocyte-activating molecules. They are used in the treatment of rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, and psoriasis.

These drugs may raise the risk of contracting tuberculosis or inducing a latent infection to become active. Infliximab and adalimumab have label warnings stating that patients should be evaluated for latent TB infection and treatment should be initiated prior to starting therapy with them.

TNF or the effects of TNF are also suppressed by various natural compounds, including curcumin (an ingredient in turmeric) and catechins (in green tea).

5.4. Mycophenolate

Mycophenolic acid acts as a non-competitive, selective, and reversible inhibitor of Inosine-5′-monophosphate dehydrogenase (IMPDH), which is a key enzyme in the *de novo* guanosine nucleotide synthesis. In contrast to other human cell types, lymphocytes B and T are very dependent on this process. Mycophenolate mofetil is used in combination with ciclosporin or tacrolimus in transplant patients.

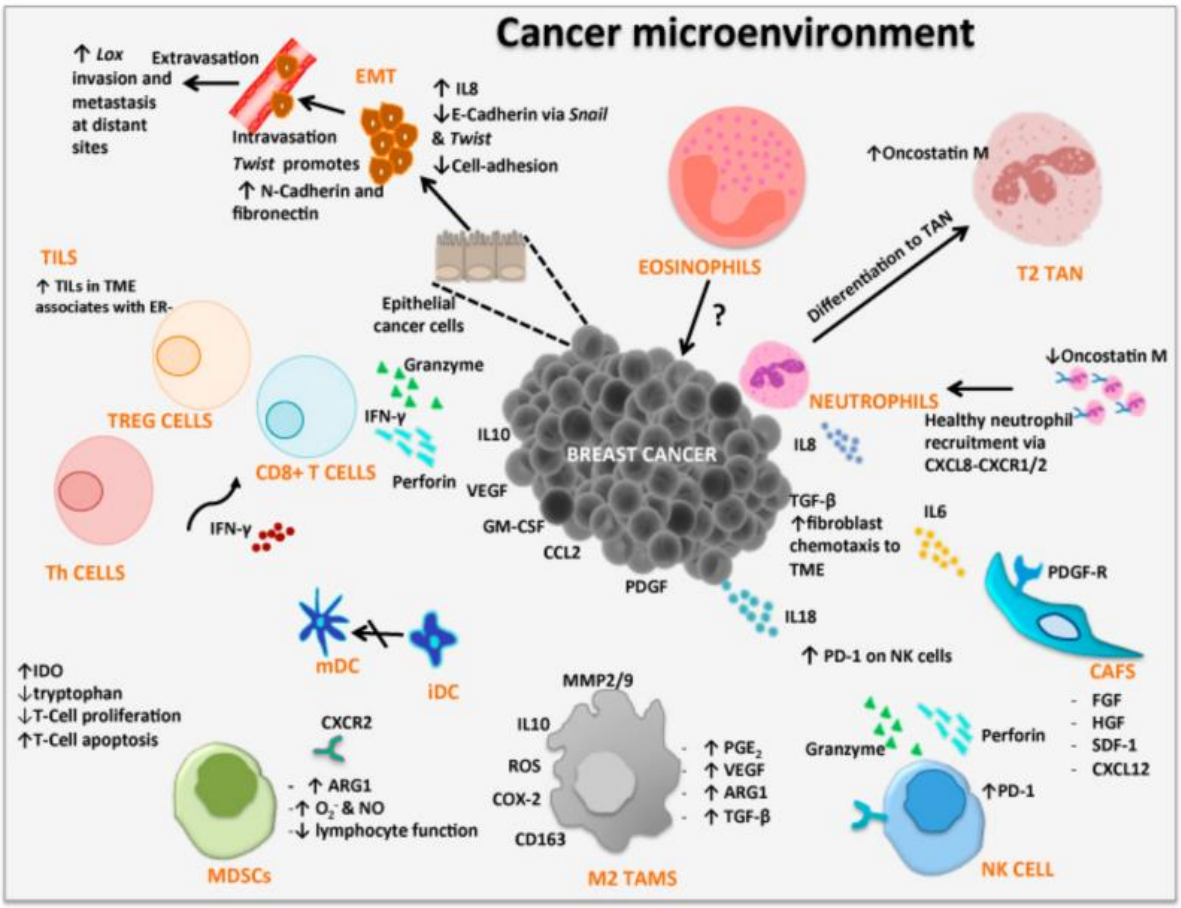
5.5. Small biological agents

Fingolimod is a new synthetic immunosuppressant, currently in phase 3 of clinical trials. It increases the expression or changes the function of certain adhesion molecules (α4/β7 integrin) in lymphocytes, so they accumulate in the lymphatic tissue (lymphatic nodes) and their number in the circulation is diminished. In this respect, it differs from all other known immunosuppressants.

Myriocin has been reported being 10 to 100 times more potent than Ciclosporin.

TUMOUR IMMUNOLOGY

Cancer immunology is an interdisciplinary branch of biology that is concerned with understanding the role of the immune system in the progression and development of cancer; the most well known application is cancer immunotherapy, which utilises the immune system as a treatment for cancer. Cancer immunosurveillance and immunoediting are based on protection against development of tumors in animal systems and (ii) identification of targets for immune recognition of human cancer.



Definition

Cancer immunology is an interdisciplinary branch of biology concerned with the role of the immune system in the progression and development of cancer; the most well known application is cancer immunotherapy, where the immune system is used to treat cancer.Cancer immunosurveillance is a theory formulated in 1957 by Burnet and Thomas, who proposed that lymphocytes act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells. Cancer immunosurveillance appears to be an important host protection process that decreases cancer rates through inhibition of carcinogenesis and

maintaining of regular cellular homeostasis. It has also been suggested that immunosurveillance primarily functions as a component of a more general process of cancer immunoediting.

Tumor antigens

Main article: Tumor antigen

Tumors may express tumor antigens that are recognized by the immune system and may induce an immune response. These tumor antigens are either TSA (Tumor-specific antigen) or TAA (Tumor-associated antigen)

Tumor-specific antigens (TSA)

Tumor-specific antigens are antigens that only occur in tumor cells.TSAs can be products of oncoviruses like E6 and E7 proteins of Human papillomavirus, occurring in cervical carcinoma, or EBNA-1 protein of EBV, occurring in Burkitt's lymphoma cells. Another example of TSAs are abnormal products of mutated oncogenes (e.g. Ras protein) and anti-oncogenes

Tumor-associated antigens (TAA)

Tumor-associated antigens are present in healthy cells, but for some reason they also occur in tumor cells However, they differ in quantity, place or time period of expression. Oncofetal antigens are tumor-associated antigens expressed by embryonic cells and by tumors.Examples of oncofetal antigens are AFP (α-fetoprotein), produced by hepatocellular carcinoma, or CEA (carcinoembryonic antigen), occurring in ovarian and colon cancer. More tumor-associated antigens are HER2/neu, EGFR or MAGE-1.

Cancer immunoediting

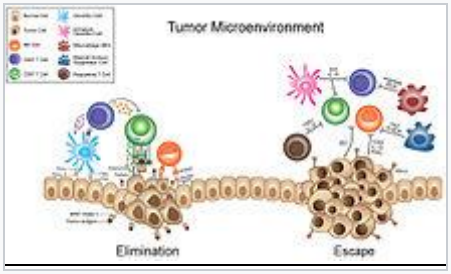
Cancer immunoediting is a process in which immune system interacts with tumor cells. It consists of three phases: elimination, equilibrium and escape. These phases are often referred to as "the three Es" of cancer immunoediting. Both, adaptive and innate immune system participate in immunoediting.

In the elimination phase, the immune response leads to destruction of tumor cells and therefore to tumor suppression. However, some tumor cells may gain more mutations, change their characteristics and evade the immune system. These cells might enter the equilibrium phase, in which the immune system doesn't recognise all tumor cells, but at the same time the tumor doesn't grow. This condition may lead to the phase of escape, in which the tumor gains dominance over immune system, starts growing and establishes immunosuppressive environment.

As a consequence of immunoediting, tumor cell clones less responsive to the immune system gain dominance in the tumor through time, as the recognized cells are eliminated. This process may be considered akin to Darwinian evolution, where cells containing pro-oncogenic or immunosuppressive mutations survive to pass on their mutations to daughter cells, which may themselves mutate and undergo further selective pressure. This results in the tumor consisting of cells with decreased immunogenicity and can hardly be eliminated.This phenomenon was proven to happen as a result of immunotherapies of cancer patients.

Mechanisms of tumor evasion from the immune response

Tumor cells have developed various mechanisms to escape immune surveillance



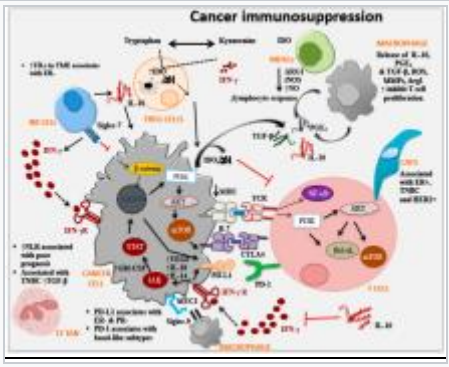
Multiple factors determine whether tumor cells will be eliminated by the immune system or will escape detection. During the elimination phase immune effector cells such as CTL's and NK cells with the help of dendritic and CD4+ T-cells are able to recognize and eliminate tumor cells.

- CD8+ cytotoxic T cells are a fundamental element of anti-tumor immunity. Their TCR receptors recognise antigens presented by MHC class I and when bound, the Tc cell triggers its cytotoxic activity. MHC I are present on the surface of all nucleated cells. However, some cancer cells lower their MHC I expression and avoid being detected by the cytotoxic T cells.This can be done by mutation of MHC I gene or by lowering the sensitivity to IFN-γ (which influences the surface expression of MHC I). Tumor cells also have defects in antigen presentation pathway, what leads into down-regulation of tumor antigen presentations. Defects are for example in Transporter associated with antigen processing (TAP) or Tapasin On the other hand, a complete loss of MHC I is a trigger for NK cells. Tumor cells therefore maintain a low expression of MHC I.
- Another way to escape cytotoxic T cells is to stop expressing molecules essential for co-stimulation of cytotoxic T cells, such as CD80 or CD86.
- Tumor cells express molecules to induce apoptosis or to inhibit T lymphocytes:

- Expression of FasL on its surface, tumor cells may induce apoptosis of T lymphocytes by FasL-Fas interaction.
- Expression of PD-L1 on the surface of tumor cells leads to suppresion of T lymphocytes by PD1-PD-L1 interaction.

- Tumor cells have gained resistance to effector mechanisms of NK and Cytotoxic CD8+ T cell:
  - by loss of gene expression or inhibiton of apoptotic signal pathway molecules: APAF1, Caspase 8, Bcl-2-associated X protein (bax) and Bcl-2 homologous antagonist killer (bak).[*citation needed*]
  - by induction of expression or overexpression of antiapoptotic molecules: Bcl-2, IAP or XIAP.

Tumor cells have developed various mechanisms to create immunosuppressive tumour microenvironment[edit]



Immune checkpoints of immunosuppressive actions associated with breast cancer

- Production of TGF-β by tumor cells and other cells (such as Myeloid-derived suppressor cell) leads to conversion of CD4+ T cell into suppressive regulatory T cell (Treg) by a contact dependent or independent stimulation. In a healthy tissue, functioning Tregs are essential to maintain self-tolerance. In a tumor, however, Tregs form an immunosupresive microenviroment.
- Tumor cells produce special cytokines (such as Colony-stimulating factor) to produce Myeloid-derived suppressor cell. These cells are heterogenous collection of cell types including precursors of Dendritic cell, Monocyte and Neutrophil. MDSC have suppressive effects on T-lymphocytes, Dendritic cells and Macrophages. They produce immunosupressive TGF-β and IL-10.
- Another producer of suppresive TGF-β and IL-10 are Tumor-associated macrophages, these macrophages have mostly phenotype of alternatively activated M2 macrophages. Their

activation is promoted by TH type 2 cytokines (such as IL-4 and IL-13). Their main effects are immunosuppresion, promotion of tumor growth and angiogenesis.[33]

- Tumor cells have non-classical MHC class I on their surface, for example HLA-G. HLA-G is inducer of Treg, MDSC, polarise macrophages into alternatively activated M2 and has other immunosuppresive effects on immune cells.

Mechanisms of immunomodulation in the fight against cancer

Immune system is the key player in fighting cancer. As described above in mechanisms of tumor evasion, the tumor cells are modulating the immune response in their profit. It is possible to improve the immune response in order to boost the immunity against tumor cells.

- monoclonal anti-CTLA4 and anti-PD-1 antibodies are called immune checkpoint inhibitors:
  - CTLA-4 is a receptor upregulated on the membrane of activated T lymphocytes, CTLA-4 CD80/86 interaction leads to switch off of T lymphocytes. By blocking this interaction with monoclonal anti CTLA-4 antibody we can increase the immune response. An example of approved drug is ipilimumab.

PD-1 is also an upregulated receptor on the surface of T lymphocytes after activation. Interaction PD-1 with PD-L1 leads to switching off or apoptosis. PD-L1 are molecules which can be produced by tumor cells. The monoclonal anti-PD-1 antibody is blocking this interaction thus leading to improvement of immune response in CD8+ T lymphocytes. An example of approved cancer drug is nivolumab.

Chimeric Antigen Receptor T cell

This CAR receptors are genetically engineered receptors with extracellular tumor specific binding sites and intracelullar signalling domain that enables the T lymphocyte activation.

Cancer vaccine

Vaccine can be composed of killed tumor cells, recombinant tumor antigens, or dendritic cells incubated with tumor antigens (dendritic cell-based cancer vaccine)

Cancer immunology and chemotherapy

cancer immunotherapy

Obeid et al.] investigated how inducing immunogenic cancer cell death ought to become a priority of cancer chemotherapy. He reasoned, the immune system would be able to play a factor via a ‘bystander effect’ in eradicating chemotherapy-resistant cancer cells. However,

extensive research is still needed on how the immune response is triggered against dying tumour cells.

Professionals in the field have hypothesized that 'apoptotic cell death is poorly immunogenic whereas necrotic cell death is truly immunogenic'. This is perhaps because cancer cells being eradicated via a necrotic cell death pathway induce an immune response by triggering dendritic cells to mature, due to inflammatory response stimulation. On the other hand, apoptosis is connected to slight alterations within the plasma membrane causing the dying cells to be attractive to phagocytic cells. However, numerous animal studies have shown the superiority of vaccination with apoptotic cells, compared to necrotic cells, in eliciting anti-tumor immune responses.

Thus Obeid *et al.* propose that the way in which cancer cells die during chemotherapy is vital. Anthracyclins produce a beneficial immunogenic environment. The researchers report that when killing cancer cells with this agent uptake and presentation by antigen presenting dendritic cells is encouraged, thus allowing a T-cell response which can shrink tumours. Therefore, activating tumour-killing T-cells is crucial for immunotherapy success.

However, advanced cancer patients with immunosuppression have left researchers in a dilemma as to how to activate their T-cells. The way the host dendritic cells react and uptake tumour antigens to present to $CD4^+$ and $CD8^+$ T-cells is the key to success of the treatment..

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