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STUDY MATERIAL

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SUBJECT PAPER	**IMMUNOLOGY**		**PAPER CODE MZO33**
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IMMUNOLOGY

UNIT-II: IMMUNOGLOBULINS

Immunoglobulins-structure, isotypes and biological function. Antigenic determinant on immunoglobulin-isotype, allotype and idiotype. Immunoglobulin superfamily, monoclonal and polyconal antibodies. organization and expression of immunoglobulin genes. Synthesis of immunoglobulin and disorders of immunoglobulin synthesis.

UNIT-III: DETECTION AND APPLICATION OF ANITGEN ANTIBODY REACTION

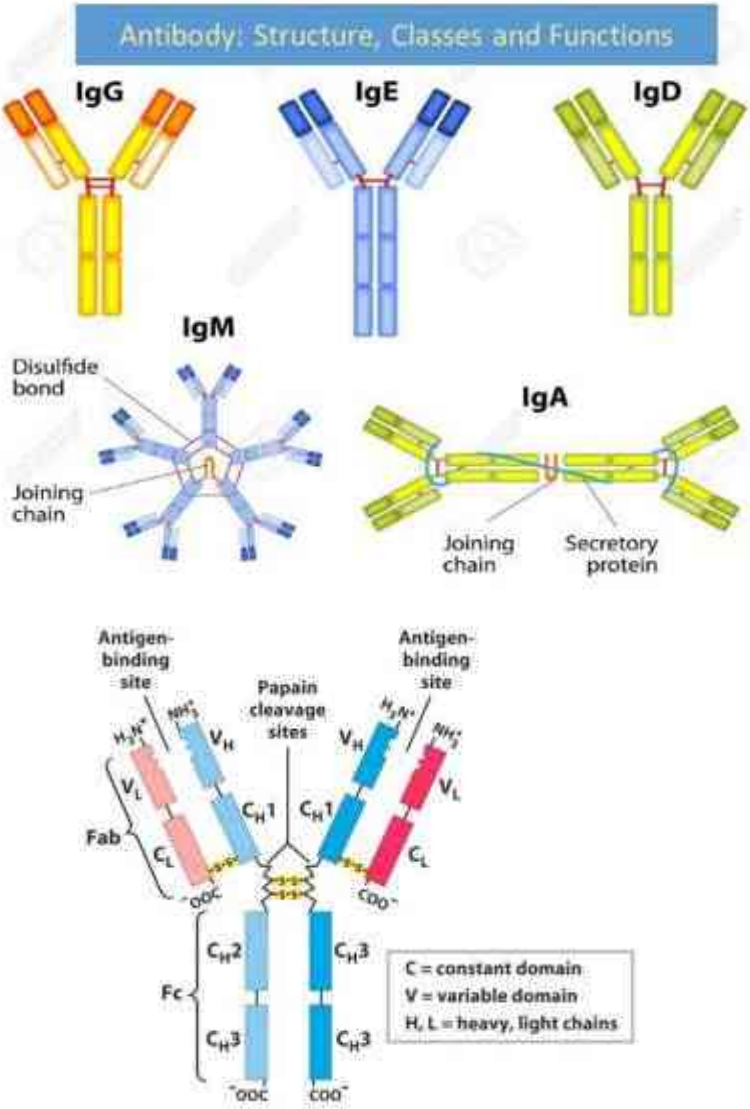
Precipitiation - agglutination - complement fixation - immunoassay using labelled reagents.

UNIT-II: IMMUNOGLOBULINS

Immunoglobulins-structure

- Antibodies are the globular protein belonging to immunoglobulin (Ig) family.
- Antibody molecules have a common structure of four peptide chains. This structure consists of two identical light (L) chain polypeptide of about 22000 Da and two identical heavy (H) chain of larger polypeptide of about 55000 Da or more.
- Each light chain is bound to a heavy chain by a disulphide bond and by non-covelent interactions such as salt bride, hydrogen bonds and hydrophobic interaction to form a heterodimer (H-L). Similar non-covalent interaction and disulphide linkage link the two identical heterodimer (H-L) to each other to from basic structure of antibody ie. Dimer of dimer.

Structure of antibody



Anatomy of light (L) and heavy (H) chain:

L- chain:

- L- chain of antibody is composed of about 220 aminoacids.
- Around 100-110 aminoacids are located at N-terminal (amino-terminal) and the aminoacids sequences varies among antibodies. This region of L-chain is known as variable (V) region.
- Remaining 110 aminoacids located at C-terminal (carboxyl-terminal) of L-chain are almost constant among antibodies. This region of L-chain is known as constant (C) region. Two types of constant region sequences are found ie. Lambda (λ) and Kappa (κ). In a particular antibody either2lambda or 2 kappa chains are present but not 1 lambda and kappa.
- In human 60% light chain are kappa and 40% are lambda whereas in mice 95% of light chain are kappa and 5% are lambda.

H-chain:

- In H-chain about 110 aminoacids are located at N-terminal which shows great variation among antibody. This region is known as Variable (V) region.
- Remaining aminoacid sequences of H-chain is somewhat constant but reveals five different types of constant (C) heavy chain region ie. μ, α, δ, ε and γ.
- The length of constant region of H-chain is 330 aminoacids for α, γ and δ and 440 aminoacids for μ and ε.

Antibodies molecules are classified into five class on the basis of constant region of H-chain.

Domain structure of antibody:

- The overall structure of immunoglobulin molecule is determined by primary, secondary, tertiary and quaternary organization of aminoacid molecules.
- The primary structure is sequence of aminoacids that comprises variable and constant region of heavy and light chain.
- The secondary structure is formed by folding of polypeptide chain into series of beta (β) pleated sheets.
- The secondary structure is then folded into tertiary structure of compact globular domains.
- Finally these globular domains of adjacent heavy and light chain interacts in quaternary structure forming functional domains that enables binding site for antigen and the same time performs a number of biological functions.
- Two domains are found in L-chain ie one in variable region (VL) and other in constant region (CL).
- In H-chain, one domain is found in Variable region (VH). In IgA, IgG and IgD three domains are found in constant region (CH1, CH2 and CH3) whereas in IgE and IgM fou domains are found in constant region of H-chain (CH1, CH2, CH3 and CH4.

Fab, Fc and Hinge region of antibody:

*1. **Fab region***

- Antigen binding is accomplished by amino-terminal (N-terminal) region and effector functions by carboxyl terminal (C-terminal) region of antibody.
- In an antibody molecule two Fab regions are found and they binds antigens.
- Hypervariable region on L-chain (VL domain) and H-chain (VH domain) form antigen binding site.
- Detailed comparison of aminoacids sequences of large number of VL and VH domain reveals that the sequence variation is concentrated in few discrete region of these

domains. The variability plot of VH and VL domains shows maximum variation in certain region which is known as hypervariable region and this forms antigen binding site.

- Antigen binding site is complementary to epitope of antigen, so it is also known as complementary determining regions (CDRs).

2.Fc region:

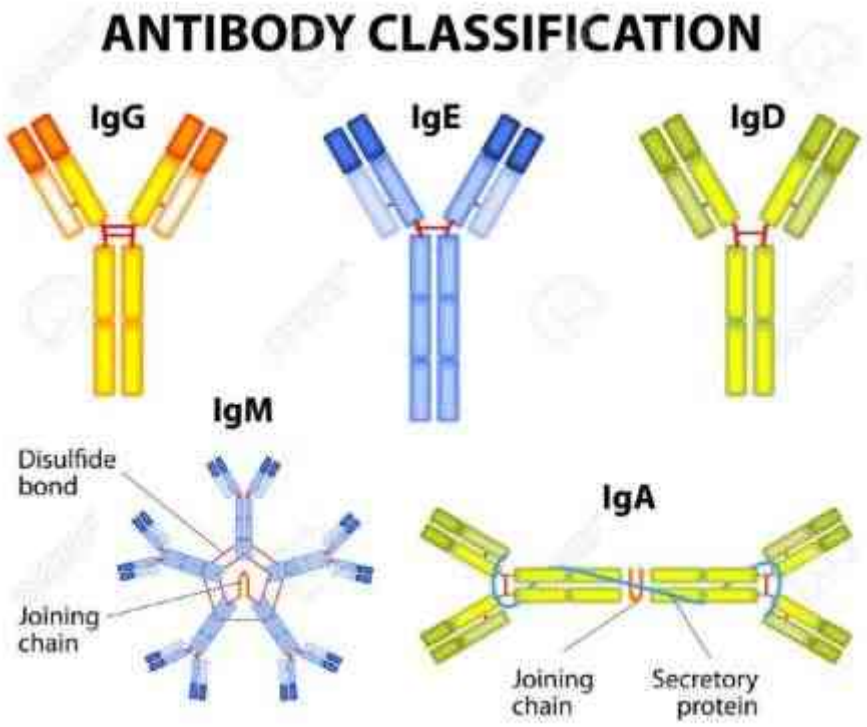
- Fc region of immunoglobulin allows for interaction of immune complex with other phagocytic cells and complement.
- Take parts in various biological functions that are determined by aminoacid sequences of each domains of constant region.
- Many different form of Fc receptors exists.

*3****. Hinge region:***

- The γ, δ and α heavy chain contains an extended peptide sequence between CH1 and CH2 domain that has no homology with other domain, this region is known as hinge region.
- Hinge region is rich in proline residue and is flexible. Therefore IgG, IgD and IgA are flexible.
- The flexibility given by hinge region enable Fab region to assume various angle to bind antigen.

Immunoglobulin classes or Isotypes

Antibody belongs to class of protein called Immunoglobulin (Ig). And classified into 5 classes or isotypes



1. IgG:

- Molecular weight: 150,000 Da

- H-chain type: gamma (53,000 Da)
- IgG is the most abundant class of Immunoglobulin in serum and constitute of about 80% of total serum immunoglobulin.
- IgG molecule consists of two gamma (γ) heavy chains and two kappa (k) or two lambda (λ) light chains.
- There are four sub class of IgG (IgG1, IgG2, IgG3 and IgG4) on the basis of decreasing serum concentration.
- It has longest half-life among other antibodies. Half-life is about 23 days.
- IgG is the only antibody that can cross placenta. It cross placenta and provide immunity to fetus upto 6 month of age. The immunity is known as natural passive immunity.
- It can also activate complement.

Biological functions:

- IgG is the major antibody produced in secondary immune response.
- Ig, IgG3 and IgG4 readily cross the placenta and play important role in protecting the fetus.
- IgG3 is the most effective complement activator followed by IgG1 and IgG2. IgG4 is not able to activate complement at all.
- IgG1 and IgG3 binds with high affinity to Fc receptor on phagocytic cell and thus mediate opsonization.
- IgG helps in bacterial immobilization.
- IgG neutralize toxin and viruses.

2**. IgM:**

- Molecular weight: 900,000 Da
- H-chain type: mu (65,000 Da)
- IgM accounts for 5-10% of total serum Immunoglobulin with an average serum concentration of 1.5mg/dl.
- IgM is secreted by plasma cell and it exists in pentameric form in which five IgM mononers are linked together by disulphide bond (J-chain).
- Due to large size, IgM is also known as millionare molecule.
- There are 10 antigen binding site (Fab) in pentameric IgM molecule but it cannot bind to 10 complete antigen due to steric hindrance.
- It is the major antibody produced during primary immune response.
- Monomeric form IgM (180000 Da) is also expressed as membrane bound receptor on B-cell.

Biological functions:

- IgM is the first antibody produced in primary immune response and it is also the first Ig produced by neonate.
- IgM has higher valency (antigen binding ste) due to its pentameric form.
- Due to pentameric form, IgG is very effective in agglutination reaction.
- IgM is more efficient than IgG in complement activation.

- IgM plays important accessory role as secretory immunoglobulin due to J-chain.

3. IgA:

- Molecular weight: 320,000 Da
- H- chain type: Alpha (55000 Da)
- IgA constitute 10-15% of total serum immunoglobulin.
- It is the predominant Immunoglobulin n external secretions such as breast milk, saliva, tears and mucus of bronchial, genitourinary and digestive tracts.
- IgA primarily exists as monomeric form but dimeric, trimeric and some tetrameric form are also present.
- IgA in blood occurs in monomeric form whereas those in body secretion occurs in dimeric or multimeric forms.
- Dimeric form of IgA contains J-chain and secretory chain. Secretory chains helps in transcytosis.
- IgA can cross epithelial layer and enter into body secretion. The process of crossing epithelial layer by IgA is known as transcytosis.
- There are two sub-class of IgA ie. IgA1 and IgA2.

Biological functions;

- IgA can cross the epithelial layer and enter into body secretion and provides local immunity in GI tracts, respiratory tract, genital tract etc
- In body secretion IgA neutralize viruses and prevent attachment on host surface.

4. IgD:

- Molecular weight: 180,000 Da
- H-chain type: Delta (70000 Da)
- IgD is present in extremely low concentration and it constitute 0.2% of total serum immunoglobulin.
- IgD together with IgM is the major membrane bound immunoglobulin expressed on mature B-cell.
- There are two sub-classes of IgD (IgD1 and IgD2)
- IgD plays important role in maturation and proliferation of B-cell.

5. IgE:

- Molecular weight: 200,000 Da
- H-chain type: epsilon (73,000Da)
- IgE accounts for 0.3% of total serum Immunoglobulin.
- IgE is also known as reagenic antibody due to its involvement in allergic reaction. IgE mediate immediate hypersensitivity reaction and responsible for symptoms like hey fever, asthma, anaphylactic shocks, etc.
- Fc region of IgE binds on blood basophils and tissue mast cells. The cross reaction with antigen to Fc region bound IgE causes degranulation of mast cell and basophils releasing histamine. Histamine is responsible for symptoms of allergy.

Biological functions

- IgE provides immunity against parasite by Antibody dependent cell mediated cytotoxicity (ADCC).
- Level of IgE antibody in blood of normal individual is very low and its level increases during parasitic infection and in allergic reactions.

ANTIGENIC DETERMINANTS OF IMMUNOGLOBULIN MOLECULES

A. Isotypes

These determinants are present on all molecules of each class and subclass of im-munoglobulin heavy chains and on each type of light chains; they are defined serologically by antisera directed against the constant regions of H and L chains. The antisera are pro-duced in animals, which, upon injection of purified human immunoglobulins, recognize the structural differences between constant regions of H and L chains. Isotypic determinants are common to all members of a given species, hence they cannot be used as genetic mark-ers. Their practical importance results from the fact that they allow the identification of classes and subclasses of immunoglobulins through the heavy-chain isotypes and types of light chains (k , λ). All classes and subclasses of normal immunoglobulins share the two light-chain isotypes.

B. Idiotypes

The antigen-combining site in the V region of the immunoglobulin molecule, in addition to determining specificity for antigen binding, can also act as an antigen and induce produc-tion of antibodies against it. Such antigenic determinants, usually associated with hyper-variable regions, are known as idiotypes.

C. Allotypes

These are hereditary antigenic determinants of Ig polypeptide chains that may differ be-tween individuals of the same species. The loci controlling allotypic determinants are codominant (i.e., both are expressed phenotypically in a heterozygote) autosomal genes that follow Mendelian laws of heredity. All allotypic markers that have so far been identi-fied on human immunoglobulin molecules, with one exception (see later), are present in the C regions of H chains of IgG, IgA, IgE, and on k-type L chains. Since different individu-als of the same species may have different allotypes, these determinants can be used as ge-netic markers.

The most common technique used for allotype determination is hemagglutination-in-hibition. For this purpose, ORh_+ red cells are coated with IgG immunoglobulins of known allotypes. The coated cells will agglutinate when

exposed to specific antibody. The agglu-tination, however, will be inhibited if the antiserum recognizing the allotype of the im-munoglobulin coating the red cell is preincubated with soluble IgG carrying the same allo-type. Thus, in a first step, the anti-allotypic antiserum and an unknown serum to be typed are mixed. In a second step, red cells coated with the relevant allotype are added to dilu-tions of the mixture. If agglutination is inhibited, it can be concluded that the allotype was present in the unknown serum.

1. IgG Heavy-Chain Allotypes (GM Allotypes)

Allotypes have been found on γ1, γ2, and γ3 heavy chains but not as yet on γ4 chains. They are denoted as G1M, G2M, and G3M, respectively (G for IgG, the numerals 1, 2, and 3 identify the subclass, the letter M for marker). At present, 18 GM specificities can be de-fined (Table 7.1): 4 associated with IgG1 (G1M), 1 associated with IgG2 (G2M), and 13 associated with IgG3 (G3M). G1M 3 and G1M 17 are localized in the Fd portion of the IgG molecule, while the rest are in the Fc portion. In some cases it has been shown that the anti-genic differences recognized as allotypic are a consequence of single amino acid substitu-tion on the heavy chains. For instance, G1M 3 heavy chains have arginine at position 214 and G1M 17 heavy chains have lysine at this position. A single heavy chain may possess more than one GM determinant; G1M 17 and G1M 1 are frequently present on the Fd and Fc portions of the same H chain in Caucasians.

Table 7.1 Currently Testable GM Allotypes

Heavy-chain subclass	Numeric	Alphameric
γ1	G1M 1	a
	2	x
	3	f
	17	z
γ2	G2M 23	n
γ3	G3M 5	b1
	6	c3
	10	b5
	11	b0
	13	b3
	14	b4
	15	s
	16	t
	21	gl
	24	c5
	26	u
	27	v
	28	g5

The four C-region genes on human chromosome 14 that encode the four IgG sub-classes are very closely linked. Because of this close linkage, GM allotypes of various sub-classes are transmitted as a group called a haplotype. Also, because of almost absolute link-age disequilibrium between the alleles of various IgG C-region genes, certain allotypes of one subclass are always associated with certain others of another subclass. For example, the IgG1 gene controls G1M 3, whereas the IgG3 gene controls G3M 5 and G3M 21. We should expect to find G1M 3 associated with G3M 5 as often as with G3M 21; in fact, in Caucasians, a haplotype carrying G1M 3 is almost always associated with G3M 5 and not with G3M 21. Every major ethnic group has a distinct array of GM haplotypes. GM* 3 23 5,10,11,13,14,26 and GM* 1,17 5,10,11,13,14,17,26 are examples of common Caucasian and Negroid haplotypes, respectively. In accordance with the international system for hu-man gene nomenclature, haplotypes and phenotypes are written by grouping together the markers that belong to each subclass, by the numerical order of the marker and of the sub-class; markers belonging to different subclasses are separated by a space, while allotypes within a subclass are separated by commas. An asterisk is used to distinguish alleles and haplotypes from phenotypes.

2. IgA Heavy-Chain Allotypes (AM Allotypes)

Two allotypes have been defined on human IgA2 molecules: A2M 1 and A2M 2. They be-have as alleles of one another. No allotypes have been found on IgA1 molecules as yet. In-dividuals lacking IgA (or a particular IgA allotype) have in some instances been found to possess anti-IgA antibodies directed either against one of the allotypic markers or against the isotypic determinant. In some patients these antibodies can cause severe anaphylactic reactions following blood transfusion containing incompatible IgA.

3. IgE Heavy-Chain Allotypes (EM Allotypes)

Only one allotype, designated as EM 1, has been described for the IgE molecule. Because of a very low concentration of IgE in the serum, EM 1 cannot be measured by hemaggluti-nation-inhibition, the method most commonly used for typing all other allotypes. This marker is measured by radioimmunoassay using a monoclonal anti-EM 1 antibody.

4. k-Type Light-Chain Allotypes (KM Allotypes)

Three KM allotypes have been described so far: KM 1, KM 2, and KM 3. (About 98% of the subjects positive for KM 1 are also positive for KM 2.) They are inherited via three al-leles—KM* 1, KM* 1,2, and KM* 3—on human chromosome 2. No allotypes have yet been found on the λ-type light chains.

5. Heavy-Chain V-Region Allotype (HV 1)

So far, HV 1 is the only allotypic determinant described in the V region of human im-munoglobulins. It is located in the V region of H chains of IgG, IgM, IgA, and possibly also on IgD and IgE.

Immunoglobulins superfamily (IgSF)

- The immunoglobulin superfamily (IgSF) is a class of proteins that are associated with the adhesion, binding and recognition processes of cells. The term "immunoglobulin superfamily"

(IgSF) initially referred to Igs and other proteins involved in the immune response and sharing the same 3D topology. The subsequent discovery of the Ig fold in proteins not functionally related to Igs led to the definition of new functional families, structurally similar to the Igs, such as that of the cytokine receptors or of the bacterial proteins containing the fibronectin type III module.

- Proteins which have similar structure to immunoglobulins are classified into IgSF. In recent years, increasing numbers of novel members of the IgSF have been identified. Membership of the IgSF of a newly identified protein is usually based on the conservation in folding and in sequence of specific features found within the Ig molecules. These criteria include domain size (-100 amino acids), the number of strands, and the general topology of the Ig domain or Ig fold. All IgSFs possess at least one immunoglobulin domain or fold.

- The IgSF superfamily is composed of surface antigen receptors, co-receptors of the immune system, cell adhesion molecules, some cytokine receptors and molecules involved in antigen presentation to lymphocytes. Most of the IgSF members play roles in the immune system. But the sperm-specific protein Izumo is essential for sperm-egg fusion.

The major functional characteristics of IgSF members have been classed in groups as follows:

Molecular transport: Antibiotic proteins actively transport chromophores through different cellular compartments. In vertebrates, hemocyanins transport oxygen molecules. The Ig receptor (Poly IgR) transports Igs through the epithelial wall.

Morphoregulation: The proteins of the extracellular matrix are involved in the architectural organization and elasticity of a large number of tissues. Fibronectin acts as an "adhesive" that conserves tissue integrity. Cell adhesion molecules exposed on the embryonic cell surfaces (such as cadherin molecules) favor the organization of these cells into differentiated tissues.

Cell phenotype markers: e.g., tumor-cell markers and surface molecules of hematopoietic cells. The latter allow us to evaluate the cell type (B or T cells of the immune system) or the state of cell differentiation (B or pre-B cells, activated T cells, transformed cells, etc.).

Cell adhesion molecules: The cellular immune response requires many cell adhesion molecules of the IgSF, such as CD2, CD4, CD8, and MHC (major histocompatibility complex molecules). Some cell phenotype markers are also involved in cell adhesion, e.g., B7, B29, CD19, CD3, CD7, etc.

Virus receptors: e.g., PVR, CD4, ICAM-1, and Bgp molecules, which are the respective receptors of poliovirus, HIV, rhinovirus, and MHV virus, in addition to their constitutive functions.

Shape recognition and toxin neutralization: The property of sequence polymorphism and structural variability of the immunoglobulin molecules allows the immune system to adapt for different antigens.

Viral and bacterial molecules: In bacteria, IgSF members are domains within enzymes. They also can be involved in pili assembly and/or synthesis. In viruses, they act as receptors of cellular mediators (interferons, interleukins, etc.), or, as surface proteins, they may enhance virus virulence and dissemination.

- **Others:** Some other original functions have been observed in the IgSF, such as the regulation of gene transcription (NF-kB), cell migration (VCAM, PECAM-1, etc.), or cell death marking (PD-1). Finally, a number of Ig-like domains do not possess any as-yet-identified biological function.

- Immunoglobulin (Ig) domain is the common feature of immunoglobulin superfamily members. This domain is named after immunoglobulin proteins with about 70-110 amino acids. Immunoglobulin domains contain a characteristic structure called Ig-fold with two sheets of antiparallel beta strands forming a sandwich-like structure by cysteine residues in the B and F strands. Immunoglobulin domains also have a complementarity determining region which decides the specificity of antibodies binding their ligands.

- There are more than 750 members have been identified in immunoglobulin superfamily which is the most populous family in human genome. Most of these protein members can be found in our products index. Creative-Biomart supplies abundant products related to immunoglobulin superfamily for scientific experiments.

Monoclonal and Polyclonal Antibodies

- The ability of animal immune systems to produce antibodies capable of binding specifically to antigens can be of use to develop probes for the detection of molecules of interest in a variety of research and diagnostic applications.
- Nearly all medical or cell biology researchers use immunochemical techniques for molecular analysis.
- Usually, all immunochemical methods depend upon the utilization of antibodies, and their effectiveness relies on the quality of the antibodies used.
- The antibody production process involves the preparation of antigen samples and immunization of laboratory or farm animals to trigger the expression of antigen-specific antibodies within the serum at a high level, which then can be recovered from the animal.
- Successful antibody production depends upon cautious planning and implementation concerning several steps and considerations.

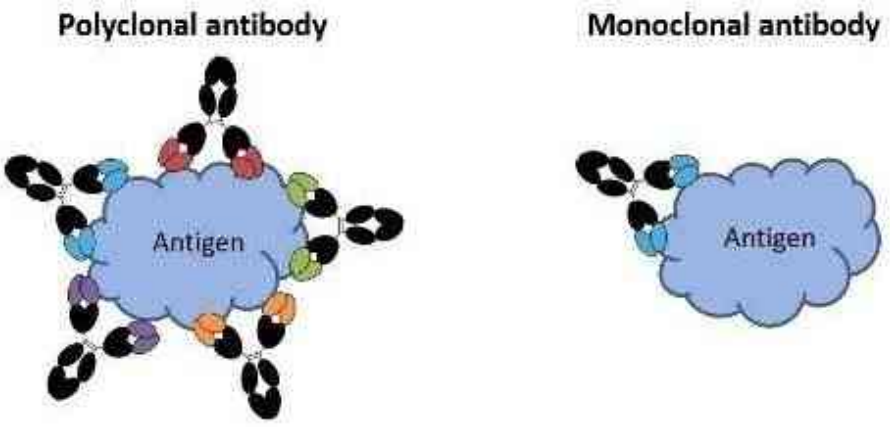


Figure: Polyclonal and Monoclonal Antibodies

Steps and Considerations of Antibody production

- Synthesis and purification of the target antigen
- Selection of an appropriate immunogenic carrier protein
- Conjugating the antigen and carrier protein to make an immunogen
- Immunization of animals using appropriate schedule and adjuvant formula
- Screening of serum or hybridoma for antibody titer and isotype

There are **two major classes of antibodies utilized in immunochemistry**:

1. **Monoclonal**
2. **Polyclonal**

Monoclonal antibodies v/s Polyclonal antibodies

Monoclonal antibodies	**Polyclonal antibodies**
Monoclonal antibodies are epitope specific for an antigen.	Polyclonal antibodies are antigen specific.
Derived from one specific clone of B cell, that recognizes one particular epitope on an antigen.	Derived from many B cell clones, that produce antibodies recognizing different epitopes of an antigen.
Produced by animal cells artificially in tissue culture using hybridoma technique.	Produced by host animal immunized with the substance of interest usually three or four times.
Expensive to produce and generally produced in mice.	Cheap to produce and generally produced in rabbits and guinea pigs for small amount, and sheep or goats for large amount.

Monoclonal antibody:

- Monoclonal antibodies are antibodies that are made by identical immune cells, clones belonging to a single parent cell.
- Monoclonal antibodies have monovalent affinity and bind to one particular epitope of an antigen.
- Monoclonal antibodies can be produced in specialized cells through a method now referred to as **hybridoma technology.**
- Kohler and Milstein in 1975, were the first to fuse lymphocytes to generate a cell line, both immortal and a producer of specific antibodies. They won the Nobel Prize for Medicine in 1984 for the development of 'hybridoma'.
- The value of hybridoma was not well acknowledged before 1987, around which the production of monoclonal antibodies in rodents for usage in diagnostics started.
- B cells can mutate into tumor cells that result in a type of cancer called myeloma. Myeloma cells are immortal and grow indefinitely in culture. The fusion of a single activated B cell and a myeloma cell creates hybridoma that can grow continuously in culture and produce antigen-specific antibodies.

Production steps of Monoclonal Antibody:

- Mice are immunized with an antigen, and their blood is screened for antibody production.
- The antibody-producing splenocytes are extracted for *in vitro* hybridoma production.
- Myeloma cells are made ready for fusion.
- Myeloma cells and isolated splenocytes are fused, creating hybridomas in the presence of polyethylene glycol (PEG). PEG causes the fusion of cell membranes[1].
- The hybridoma clones can be selected using **HAT** medium. Clones are screened and selected based on antigen specificity and immunoglobulin class.
- Each positive clone is confirmed, validated, and characterized (Isotyping).
- Positive clones are expanded, scaling up the production of the desired antibodies.

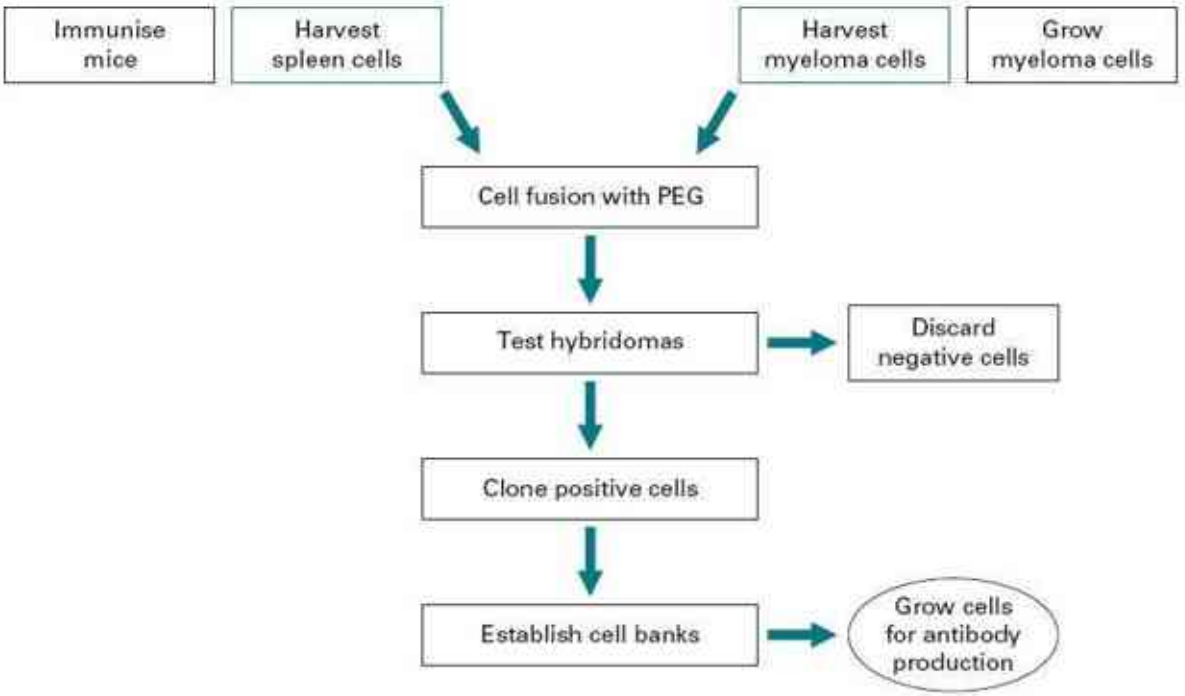


Fig. 7.5 Monoclonal antibody production.

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- This makes the cells depend upon another pathway that needs HGPRT (Hypoxanthine-guanine phosphoribosyl transferase) enzyme for DNA synthesis.

- Myeloma cells which do not fuse with the B cells cannot grow in HAT medium, since they are HGPRT negative[2].
- B cells that are not fused with the myeloma cells also die as they have short life-span.
- Therefore, the HAT medium allows the selection of hybridoma cells with the HGPRT gene and immortal property.

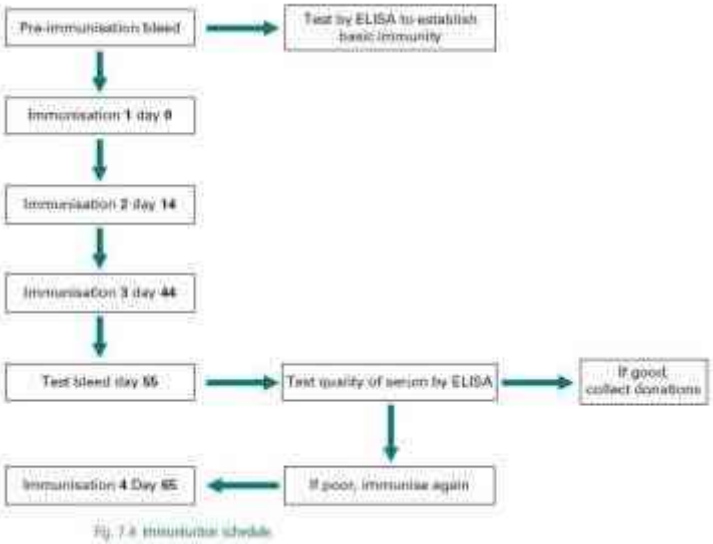
Polyclonal antibody:

- Polyclonal antibodies are antibodies that are secreted by different B cell lineages within the body.
- They are a collection of antibodies that react against a particular antigen, each binding to different epitopes.
- Polyclonal antibodies are produced in the appropriate donor animals.
- Usually, antigens are conjugated with an adjuvant before immunizing the animals.
- **Adjuvants** are substances that increase the immunogenicity of the antigen, reducing the amount of antigen required as well as stimulating specific immunity to it.
- **Freund's complete adjuvant (FCA)**, alum, bentonite, *and Bacillus pertussis* are some of the adjuvants that can be of use.

Production steps of Polyclonal Antibody:

- Preimmunize blood samples are collected to produce baseline IgG levels.
- The first two immunizations are done within 14 days.
- Later immunizations are spaced at intervals of 4-6 weeks to maximize the antibody production.
- Blood samples are collected 10 days after the completion of immunization program.
- The serum screened for presence of antibodies with specific activity to antigen. Method such enzyme-linked immunosorbent assay (ELISA) can be used for the activity testing.

Figure: Production of polyclonal antibodies



ORGANIZATION AND EXPRESSION OF IMMUNOGLOBULIN GENES

- Define the following terms: allelic exclusion, isotype switching, affinity maturation, antibody repertoire, alternative RNA splicing, recombination signal sequence Describe the genes that encode Ig Heavy and Light chains. Describe the sequence of Ig gene rearrangement that

occurs during B cell differentiation. Discuss the mechanisms of heavy chain class switching. Calculate the number of possible Igs which can be produced from a given number of V, J, D, and C genes

- DILEMMA Since only 31-35 thousand genes in the human genome actually encode proteins, How then is antibody diversity (between 1 million to 100 million specificities) achieved with such a limited number of genes?
- Key Contributor S. Tonegawa
- Properties of Antibodies The vast diversity of antibody specificities The presence in Ig heavy and light chains of a variable region at the amino-terminal end and a constant region at the carboxyl-terminal end The existence of isotypes with the same antigenic specificity, which result from the association of a given variable region with different heavy-chain constant regions
- Organization of Immunoglobulin Genes Numerous V region genes are preceded by Leader or signal sequences (60-90 bp) exons interspersed with introns. Heavy chain contains V (Variable), D (Diversity), J (Joining) and C (Constant) region gene segments. – V - D - J – C Light chain contains V, J, and C region gene segments – V - J - C Constant region genes are sub-divided into exons encoding domains (CH1,CH2, CH3, CH4)
- Kappa Light Chain Gene Organization and Rearrangement
- Heavy Chain Gene Organization and Rearrangement
- MECHANISM OF IMMUNOGLOBULIN RE-ARRANGEMENT Occurs principally via looping out (excision) of intervening gene sequences followed by ligation of Ig gene segments. Controlled by recombination signal sequences (RSS) located at joining sites. Consist of heptamer/nonamer (7/9) sequences interspersed by 12/23 base pair spacers. Recognized by Recombinases (enzymes with endonuclease and ligase activities). Consists of RAG1,2 proteins (lymphocyte-specific, and nonlymphocyte- specific DNA repair proteins (DNA ligase IV, DNAdependent protein kinase (DNA-PK) and Ku, a protein that associates with DNA-PK Genes encoding recombinases are present in all cell types but are expressed only in lymphoid (B &T) cells. Recombination activating genes 1 and 2 (RAG-1, RAG-2) have been identified which stimulate Ig gene rearrangement. Have endonuclease activity
- Antibody Diversity Mechanisms To date, seven means of antibody diversification have been identified in mice and humans: – 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

- MECHANISMS FOR GENERATING ANTIBODY DIVERSITY Junctional Diversity – Imprecise joining – N/P region (insertional) diversity occurs in VDJ joining (heavy chain) as well as VJ join of light chain. Arises from addition of up to 20 nucleotides by terminal deoxynucleotidyl transferase (TdT).
- Somatic Hypermutation – Occurs randomly after antigenic stimulation and principally in CDR1, CDR2, CDR3 regions (more frequent in CDR3). – Introduces point mutations at a higher rate than for normal mammalian genes. – Mutation rate of V genes is 1 base pair change per 103 base pairs/cell division; it is 10-7 in other mammalian genes. – Can give rise to Ig with different (new) antigen specificities leading to high or low affinity Abs. High affinity B cell clones are selectively expanded (Affinity Maturation). – Affinity maturation is associated with isotype switching.
- ISOTYPE SWITCHING Is the conversion of an immunoglobulin from one isotype to another (e.g. IgG to IgE) while retaining the same antigen specificity. Switching is dependent on antigenic stimulation and is induced by cytokines released by helper T cells and requires engagement of CD40L e.g. IL-4 triggers switching from IgM to IgE or IgG4 (humans); IFN-γ triggers switching from IgM to IgG2a (mice)]. Cyokines are thought to alter chromatin structure making switch sites more accessible to recombinases for gene transcription. Involves switch sites located in introns upstream of each CH segment (except Cδ). Class switching occurs usually in activated B cells (including memory cells) and not in naïve B cells and involves heavy chain genes. These cells (you will recall) already have rearranged VDJ genes at the DNA
- **Variable-Region Gene Rearrangements**

  V-J rearrangements of light chainsV-D-J rearrangements of heavy chains
- **Mechanisms recombination signal sequences**

  enzymatic joining of gene segementsRAG-1 RAG-2DefectsProductive vs non productive gene rearrangementsAllelic exclusion
- **Generation of Antibody Diversity**

  multiple germ line gene segmentscombinatorial V-J & V-D-J joiningjunctional flexibilityP-nucleotide additionN-nucleotide additionSomatic hypermutationAssociation of light & heavy chains
- **Class Switching in C Region Genes**

  Expression of Ig GenesDifferential processing of RNA transcripts of heavy chainsMembrane bound vs secreted IgCoexpression of IgM & IgDSynthesis, assembly and secretion

SYNTHESIS OF IMMUNOGLOBULIN

- Antibody (Ab) is also known as an immunoglobulin(Ig). These are large, Y-shaped blood proteins produced by plasma cells. They bind to foreign particles and invade them. These particles are foreign bodies that get attacked by Antibody.
- Antigens are foreign pathogens that invade the body and have the capability to give rise to a response from our immunity system either by grouping up with a larger molecule or alone after binding with antibodies for a particular immune response. Hence, antigens stimulate the production of antibodies by the immune system.

Mechanism of Antibody

- Whenever an organism's immune system encounters a foreign particle for the first time, macrophages interfere and capture to break them down so as to pass them to B cells. Once these antigens are presented, B cells begin production of a new antibody which would contain a unique paratope (site at which antibody binds with antigen) to bind with a specific epitope (site in the antigen that binds with antibody). Each lymphocyte of B cells generates a unique antibody against a unique epitope. Once the encoding is done by B cells, it releases antibodies which then bind with specific pathogens resulting in their elimination from our bodies.
- This is achieved either by direct attack of antibody on pathogens (usually when pathogens are viruses) or by binding to pathogen's surface (when the pathogen is a bacteria) and sending signals to the rest of the immune system to eliminate the pathogen. These cells remain in the body forever, ready to attack, should they re-enter the body.

Determine predominant immunoglobulin isotypes in serum.

- ✓ Determine the predominant immunoglobulin isotypes in secretions.
- ✓ Determine the predominant immunoglobulin involved in allergy.
- ✓ Determine the location of the IgD immunoglobulin.
- ✓ Determine the serum concentrations of human IgM, IgA, IgG, IgE, and IgD immunoglobulins.
- ✓ Determine which immunoglobulin is predominant in the primary, and which is predominant in secondary immune response?
- ✓ Determine which immunoglobulin isotype can cross the placenta?
- ✓ Determine which immunoglobulin isotype protect the gastrointestinal tract of the new born baby in the early life, and where it found?
- ✓ Determine the major opsonizing immunoglobulin.
- ✓ State the two different types of human IgA.
- ✓ Determine which is important in the mucosal surfaces IgA1 or IgA2.
- ✓ Identify the predominant serum IgA, and indicate in which form it exists.
- ✓ Enumerate the locations of the sIgA
- ✓ Describe the secretory component and indicate its functions.
- ✓ Identify structurally largest immunoglobulin and its locations.
- ✓ Enumerate the different functions of the IgM immunoglobulin.
- ✓ Determine the one antibody is made for ABO blood group.
- ✓ Identify the predominant antibody produced by the fetus.

- ✓ Determine the most efficient antibody in activating complement system.
- ✓ Determine which antibody which is not opsonic but enhances the opsonization process 1000-fold more than the IgG, and indicate how
- ✓ Enumerate the functions of the immunoglobulin IgE.
- ✓ Enumerate the different immunoglobulin cell surface receptors on body cells.

DISORDERS OF IMMUNOGLOBULIN SYNTHESIS

1. Osteoarthritis

It is the most common disease of joints. It is caused due to wear and tear of cartilage present between joints. It results in pain, stiffness and swelling. Osteoarthritis is more common in joints of knee, spine, hands and hip. Risk factors include age, gender, obesity, injury or overuse of joints, etc.

2. Rheumatoid arthritis

Rheumatoid arthritis is an autoimmune disorder. Here, one's own immune system attacks the healthy cells causing inflammation and pain in joints. It is more common in the joints of fingers, wrist and knee. It may also cause mild fever, anaemia and fatigue other than pain, stiffness and swelling. Inflammation can also be seen in small arteries and pericardium. In most cases of rheumatoid arthritis, a rheumatoid factor is present in the blood, which reacts with other classes of immunoglobulins.

3. Gout

Gout or gouty arthritis is a type of metabolic disorder. It is caused due to high levels of uric acid in the body or hyperuricemia.

Uric acid crystals get deposited in joints. It causes pain and inflammation. It also leads to deformities.

There is acute inflammation in one of the joints at a time, mostly in joints of extremities or big toe.

4. Bursitis

It is the inflammation of the synovial bursa, that is the fluid-filled sac present between the joints, muscles and tendon for cushioning. It causes irritation, swelling, tenderness of joints.

It may be caused due to infection, autoimmune diseases or injury. It is often caused due to overuse and excessive movement, mostly due to occupational work. Shoulders, knees and elbows are commonly affected areas.

5. Infectious Arthritis

Arthritis may also be caused by various microorganisms such as bacteria, fungi and viruses. Staphylococci, pneumococci can damage joint cartilage by pus formation. Tuberculosis of the joints can cause a lot of destruction in the adjacent bones.

Some fungi also cause joint inflammation. Some of the viruses such as Rubella virus also causes joint inflammation.

UNIT-III: DETECTION AND APPLICATION OF ANITGEN ANTIBODY REACTION

Precipitiation - agglutination - complement fixation - immunoassay using labelled reagents.

Precipitation

Definition

It is a chemical reaction in which you mix two solutions of two ionic substances and a solid ionic substance (a precipitate) forms.

For example, precipitation occurs when a part of the atmosphere saturates itself with water vapour and when the right temperature comes its condenses and precipitates. The two processes which make the air saturated are the cooling of air molecules and the addition of water vapour.

Types of Precipitation

Precipitation plays a major part in the water cycle as it is the one which brings in the deposit of freshwater on the planet. It can be divided into three categories depending upon the form such as:

- *Liquid water*
- *Ice*
- *Liquid water freezing when comes in contact with the surface*.

Examples of Precipitation

Depending on the forms we could witness precipitation in various forms:

In Liquid Form precipitation occurs in:

- *Drizzle*
- *Rain*

When the above comes in contact with the air mass in subfreezing temperature it becomes

- *Freezing Rain*
- *Freezing Drizzle*

The frozen forms of precipitated water include:

- *Snow*
- *Ice Needles*
- *Hail*
- *Graupel*
- *Sleet*

Classification of Precipitation

Precipitation of water vapour can be classified into different things and have different methods of formation and few of them are

1. Raindrop

When water droplets combine each other to form bigger water droplets and when water droplets freeze onto a crystal of ice, this process is known as coalescence. The rate of the fall is considered to be negligible, that is the reason behind the clouds not falling of the sky.

Precipitation is only possible when those will form into larger drops by coalescence by the help of turbulence in which water droplets collide, producing even larger droplets. Eventually, the droplets descend and become heavy with coalescence and resistance and finally fall as rain.

2. Snowflakes

Snow crystals form when the temperature freezes the tiny cloud droplets and because water droplets are more in number than ice crystals, the crystals can grow in size at the expense of water droplets as the water vapour causes the droplets to evaporate. These droplets fall from the atmosphere due to their mass as snowflakes.

3. Hail

Like other precipitation techniques, hail forms in the storm clouds when supercooled droplets come in contact with dust and dirt. The storm's up draft blows the hailstones up and lifted again after the updraft dissipates.

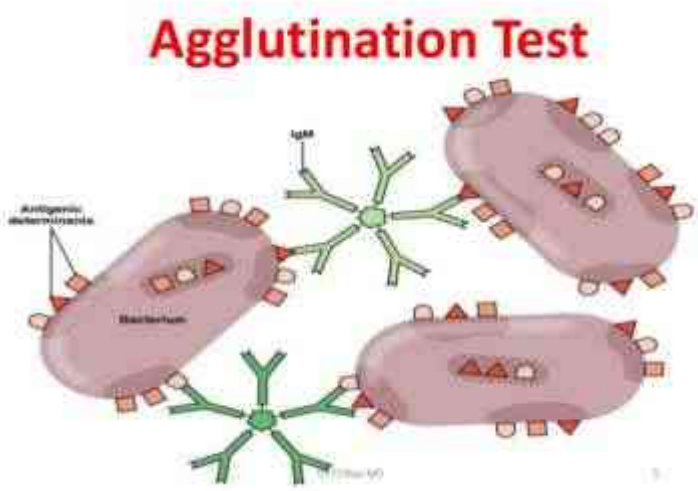
In meteorology, any result of atmospheric water vapour condensation that comes under cloud gravity is precipitation. The main types of precipitation include drizzle, rain, sleet, snow, ice pellets, graupel, and hail. Precipitation happens when water vapour (reaching 100 per cent relative humidity) saturates a portion of the atmosphere so that the water condenses and 'precipitates' or falls. Fog and mist are thus not precipitation, but colloids, since the vapour of water does not condense enough to precipitate. Two processes may contribute to air being saturated, likely working together: cooling the air or adding water vapour to the air. As smaller droplets coalesce through collision with other rain drops or ice crystals within a cloud, precipitation forms. Quick, heavy bursts of rain are called "showers" in scattered areas.

Precipitation is a significant component of the cycle of water and is responsible for the accumulation of much of the planet's fresh water. Per year, about 505,000 km3 (121,000 mi3) of water falls as precipitation, 398,000 km3 (95,000 cu mi) of it over the oceans. Given the surface area of the Earth, this means that the annual precipitation is 990 millimetres (39 in) globally averaged.

Agglutination

Definition: Two types of antigens are found on the surface of red blood corpuscles of man, antigen A and B. To react against these antigens two types of antibodies are found in the blood plasma which are accordingly known as antibody – *anti-A or a* and *anti-B or b*. Agglutination takes place only when *antigen A* and *antibody a* occur together or *antigen B* and *antibody b* are present in the blood.

- Under such condition *antibody a* reacts with *antigen A* and makes it highly sticky. Similarly *antigen B* in presence of *antibody b* become highly sticky with the result RBC's containing these antigens clump to form a bunch causing blockage of the capillaries. Agglutination in blood is therefore antigen-antibody reaction.
- 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 PH and temperature. Such a reaction is called agglutination. Antibodies that produce such reactions are called **agglutinins**.



- 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.

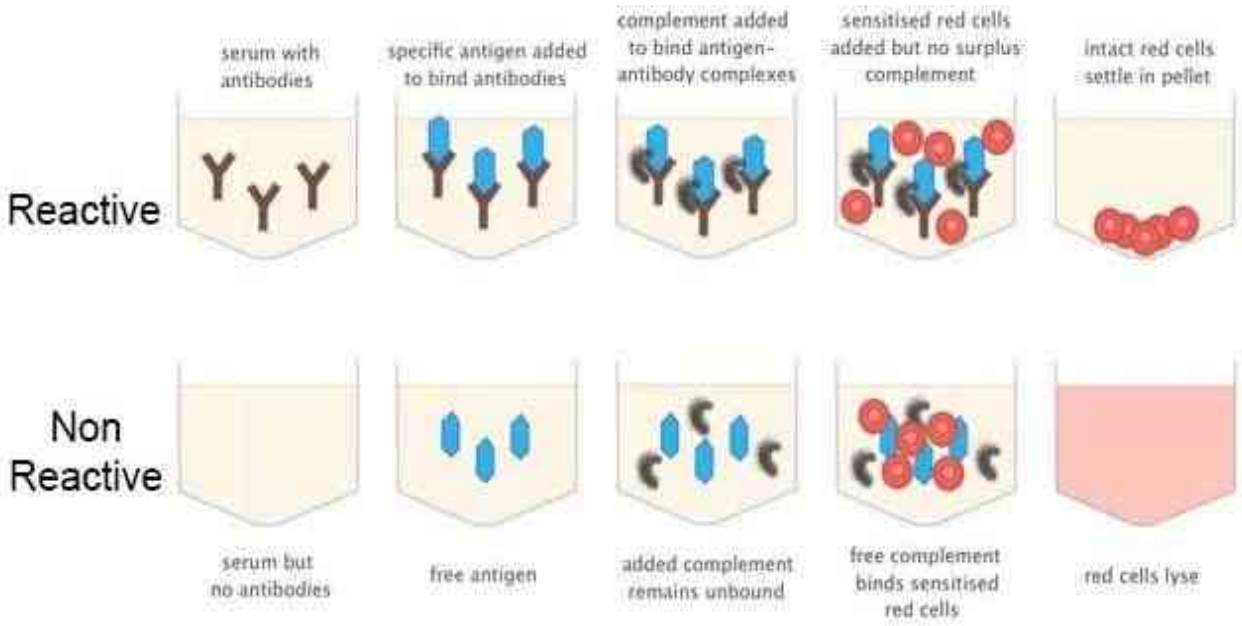
- Agglutination differs from precipitation reaction in that since agglutination reaction takes place at the surface of the particle involved, the antigen must be exposed and be able to bind with the antibody to produce visible clumps. In agglutination reactions, serial dilutions of the antibody solution 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.

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.

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.

IMMUNOASSAY USING LABELLED REAGENTS.

- **Immunoassays** have been widely used in many important areas of pharmaceutical analysis such as diagnosis of diseases, therapeutic drug monitoring, clinical pharmacokinetic and bioequivalence studies in drug discovery and pharmaceutical industries.
- Immunoassays are used in the food industry to test the raw materials as well as the final composition of food. They are also used to test for any contamination that may have occurred, as well as to test for allergens.
- Potential contamination can occur during production and transportation. Contaminants tested for include toxins, micro-organisms and pesticides.
- Allergens are tested for both known and unknown allergens. Known allergens are recorded on food labels, but sometimes food can be recalled due to accidental contamination with unexpected allergens.

❖ Foodborne Pathogen Testing

- Sandwich assays are commonly used for foodborne pathogen testing of *E. coli* and *Listeria* using polyclonal antibodies. A major problem with analyzing foodborne pathogens using polyclonal antibodies is that they have multiple epitopes. Due to this the specificity and sensitivity can be low and cause false positive results. Monoclonal antibodies are preferred and can be used to test for pathogens such as *L. monocytogenes*, *L. innocua, S. Typhimurium* and *E. coli*.
- Bispecific antibodies that recognize red blood cells and *L. monocytogenes* have been specifically engineered. The bispecific antibodies caused agglutination of red blood cells only in the presence of *L. monocytogenes*, and the agglutination shows red colored clumps that are visible just by looking with the human eye.
- Manmade single-stranded DNA molecules known as DNA enzymes are used to test for *Candida albicans*. A DNA RNA chimeric substrate at a single ribonucleotide junction is flanked by fluorophore

and a quencher. When the fluorophore and quencher separate, there is an increase in fluorescence which can then be detected.

Mycotoxin Testing

- Mycotoxins are naturally occurring toxins produced by certain molds and they are hazardous to human health. The dangerous toxins include aflatoxins, ochratoxin A, deoxynivalenol, zearalenone, and fumonisins. Mycotoxins can affect agricultural products such as corn, wheat, rice, oats, rye, soy, nuts, fruit and spices. Food and feed used can become contaminated with mycotoxins before being harvested, the time between harvesting and drying and when being stored. ELISA methods to detect mycotoxins have been available for over a decade. The most frequent way to test for mycotoxins by immunoassay is by using a direct competitive ELISA.

Allergen Testing

- Allergen testing for food is crucial because allergic reactions caused by food can cause fatality. Food allergies cause abnormal responses to the proteins that certain foods contain and cause an immune response in specific individuals. Reactions occur from IgE antibody-mediated and cell-mediated reactions. Common allergens that are tested for include milk, eggs, nuts, sesame seeds, wheat and crustacea, and multiple testing kits are available for both home and industrial use.
- Testing of final food products and swabs from equipment used in production can be used to verify any cross contamination and make sure companies are following regulations. Immunological testing methods can use human IgE from individuals allergic to specific foods, or animal IgG and IgY from polyclonal or monoclonal animal antisera. The polyclonal antisera is raised against specific proteins or mixtures of proteins, whereas the monoclonal antibodies are made specific to a particular protein or peptide.
- Qualitative detection of food allergens can be done by using dot immunoblotting or SDS-PAGE, but Rocket immunoelectrophoresis and ELISAs are used for quantitation of hidden food allergens. ELISAs are used to detect proteins that are intact, but some ELISAS cannot detect protein hydrolysate. Lipophilic residues are difficult to detect, so improvement is needed for residual detection via ELISA.

Types of Immunoassay

There are five main types of immunoassay. These include:

1. Enzyme linked immunosorbant assays (ELISA)

ELISAs work by using enzymes linked to antibodies that give a color change when in the presence of certain antigens. The antigen is immobilized on a solid surface and is then complexed with the antibody that is linked to the enzyme. ELISAs can be direct, indirect, sandwich or competitive ELISAs.

2. Radioimmunoassays

Radioimmunoassays work by using radioactively labeled antigens or antibodies. Radioactivity emitted by bound antibody-antigen complexes is detected and recorded.

3. Fluoroimmnoassays (FIA)

Fluroimmunoassays work by using antibodies that have been labeled with fluorescent probes. Fluorescence from the antibody-antigen complex is detected and recorded.

4. Chemiluminescence immunoassays (CLIA)

Chemiluminescence immunoassays work in a similar way to ELISAs, but they measure light emitted from a chemical reaction.

5. Counting Immunoassays (CIA)

Counting Immunoassays use polystyrene beads that are coated with multiple antibodies complementary to the target antigen.