

Immunology

Immunoglobulins: Structure and Function

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INTRODUCTION

The immunoglobulins (also referred as Ig) or antibodies are a group of glycoproteins present in the serum and tissue fluids of all mammals. Antibodies are produced by B cells on interaction of membrane antibody with antigens. Secreted antibodies circulate in the blood; and serve as the effectors of humoral immunity by neutralizing antigens or marking them for elimination. They therefore also constitute an element of the adaptive immune system.

Secreted antibodies circulate in the blood stream where they acts as the effectors of humoral immune response by searching and neutralizing or eliminating antigens. The immunoglobulins are present in highest concentration and most easily obtained in large quantities from blood serum. The serum antibodies produced in response to a particular antigen are heterogeneous because most antigens have many different antigenic determinant (epitopes) hence immune response produce antibody specific to each of the epitope.

Basic properties of Antibody

Antibodies, like other proteins are characterized by features such as solubility in strong salt solutions, electrostatic charge and isoelectric point, molecular weight, antigenicity (Table 1).

Solubility in salt solutions

Antibody proteins are precipitated when serum is treated with an equal volume of a saturated solution of ammonium sulfate. These proteins are classified as globulins or antibodies; the proteins that remained in the solutions were called as albumins. These specific proteins having antibody activity are called as Immunoglobulins, which is abbreviated as “Ig”.

Relative Molar Mass

Most antibodies are between 150,000 molar mass (M) to 180,000 molar mass (M) per monomer unit (Table- 1). The largest are about 900,000 M and are pentamer of a basic unit (IgM). The relative size of large protein molecule can be estimated by ultracentrifuge also.

Table 1. - Major human immunoglobulins isotypes (or classes) and their subclasses with description of its different physicochemical properties.

Immunoglobulin	IgG ₁	IgG ₂	IgG ₃	IgG ₄	IgM	IgA ₁	IgA ₂	sIgA	IgD	IgE
Heavy chain	$\gamma 1$	$\gamma 2$	$\gamma 3$	$\gamma 4$	μ	$\alpha 1$	$\alpha 2$	$\alpha 1$ or $\alpha 2$	δ	ϵ
Mean serum concentration (mg/ml)	9	3	1	0.5	1.5	3	0.5	0.05	0.03	0.00005
Sedimentation constant	7S	7S	7S	7S	19S	7S	7S	11S	7S	8S
Approx. Molecular weight (Da)	146,000	146,000	170,000	146,000	970,000	160,000	160,000	385,000	180,000	188,000
Heavy chain molecular weight	51,000	51,000	60,000	51,000	65,000	56,000	52,000	52 to 56,000	69,700	72,500
Number of domains in heavy chain	4	4	4	4	5	4	4	4	4	5
Carbohydrate	2 to 3	2 to 3	2 to 3	2 to 3	12	7 to 11	7 to 11	7 to 11	9 to 14	12
Electrophoretic mobility	γ				β	$\beta - \gamma$			γ	$\beta - \gamma$
Heavy chain	γ				μ	α			δ	ϵ
Subisotypes	$\gamma 1, \gamma 2, \gamma 3, \gamma 4$				None	$\alpha 1, \alpha 2$			None	None
Light chain	κ or λ				κ or λ	κ or λ			κ or λ	κ or λ
Half life (days)	21				5	6			3	2
Concentration in human serum (mg/ml)	800	400	100	40	100	350	40	-	3	0.01

ISOLATION & CHARACTERIZATION OF IMMUNOGLOBULINS

Significance of gamma (γ) globulin fraction

The first evidence that antibodies were contained in particular serum protein fractions came from a classical experiment by A. Tiselius and E.A Kabat (1939).

They immunized the rabbits with ovalbumin (the albumin of egg white); then divided the treated rabbit serum into two parts (Figure- 1). Electrophoresis of one serum part revealed four peaks; representing albumin and alpha (α), beta (β), and gamma (γ) globulins. The other serum part or aliquot was treated with ovalbumin protein (that acted as the antigen) some of the protein reacted with ovalbumin while the remaining protein in the aliquot which did not reacted with the antigen was electrophoresed. The results revealed that there was a significant drop in the gamma (γ) globulin peak in the second aliquot which was reacted with ovalbumin antigen (Figure- 2). Thus the gamma globulin fraction was identified to contain serum antibodies called immunoglobulins.

It is now known that Immunoglobulins G (IgG) is mainly found in significant amount in the gamma globulin fraction, and other class of antibodies are found in the alpha and beta fractions.

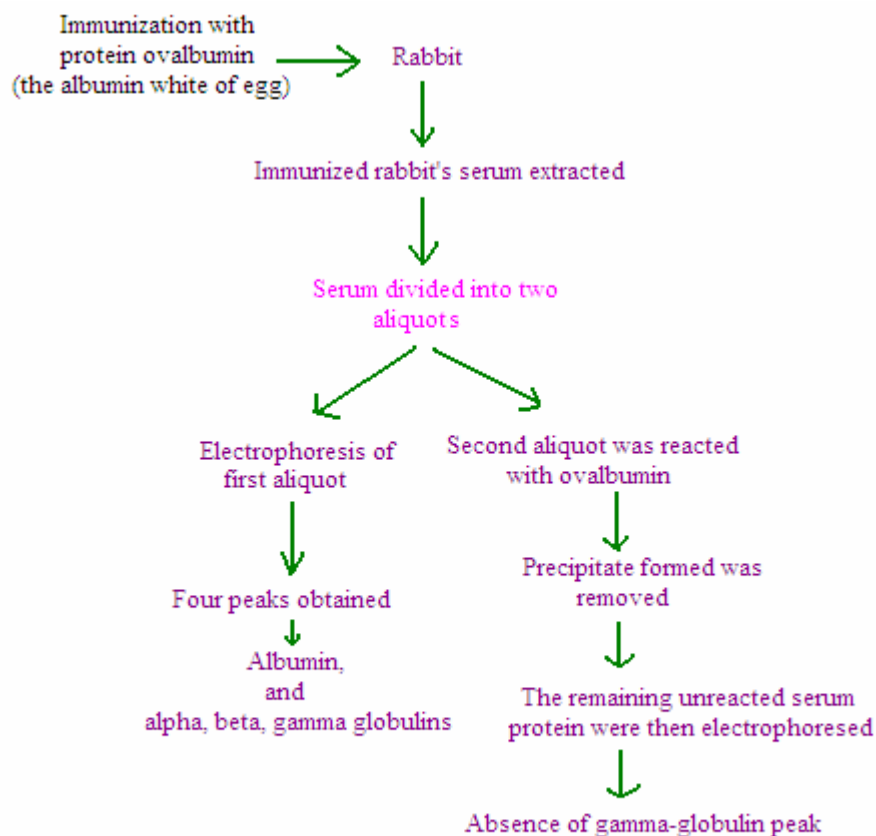


Figure 1- Overview of the classical experiment performed by A. Tiselius and E.A. Kabat (1939) to demonstrate that antibodies were contained in particular serum protein fraction.

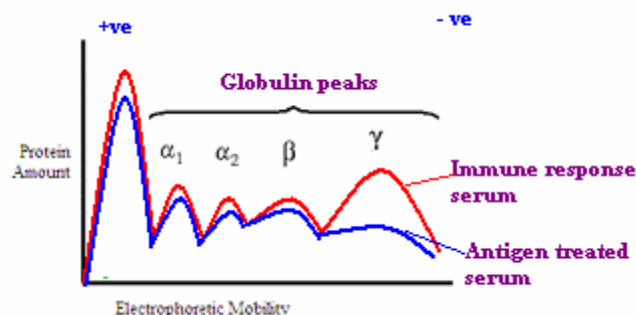


Figure 2- Comparison of the electrophoretic profiles of two serums aliquots from the experiment performed by A. Tiselius and E.A. Kabat (1939) to demonstrate that antibodies are present in the gamma (γ) globulin fraction of serum protein. The red line shows electrophoretic pattern of untreated serum while blue line shows the pattern of antisera that was treated with ovalbumin (OVA) to remove anti-OVA antibody and then electrophoresed.

Basic structure of Immunoglobulins

Antibodies are globular proteins called Immunoglobulins (Ig). They are built of two types of polypeptide chains, the larger called as heavy chain (H) with molecular mass of about 50,000 to 70,000 Daltons and the smaller one called as light chain (L) with molecular mass of about 25,000 Daltons. Two identical light chains and two identical heavy chains are present in each antibody monomer unit. Each light chain is bound to heavy chain by a disulfide bond. Each heavy and light chain (H-L) is further combined with the other heavy and light chain (H-L) by disulfide bond, to form the basic structure of immunoglobulins (Figure- 3). Henceforth each immunoglobulin contains dimer of (H-L)₂.

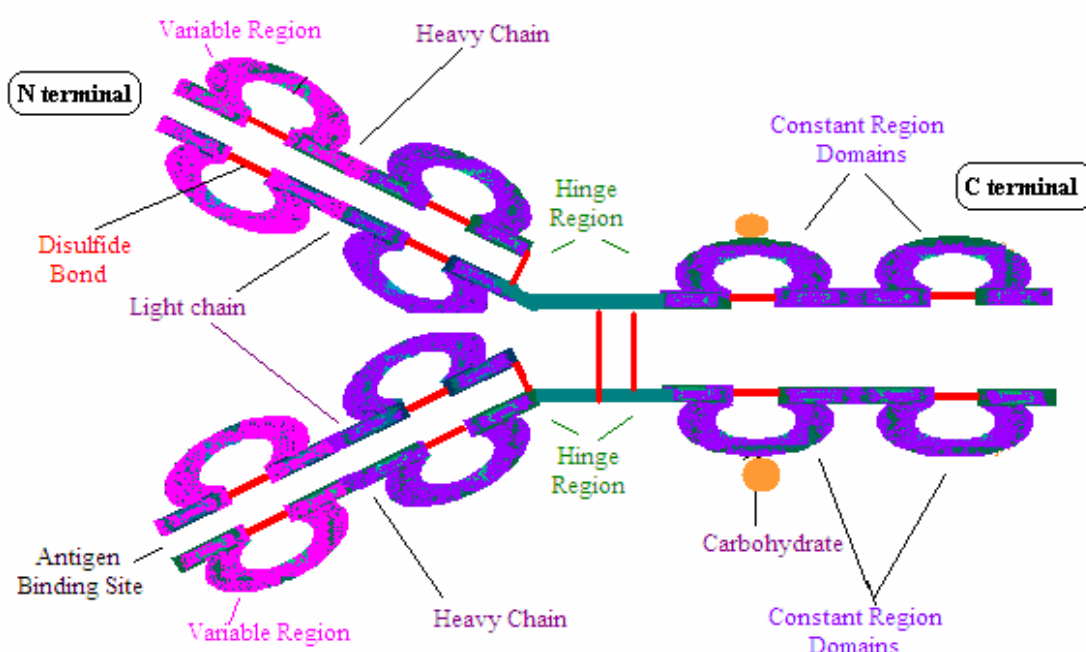


Figure 3- Basic structure of Immunoglobulin molecule (IgG). Each heavy chain and light chain contains variable region (V_H or V_L) at N terminal (pink) and the remaining chain (purple) represents constant region (C_H). IgG, IgD, and IgA contains flexible hinge region (green) mainly rich in proline residues between C_H1 and C_H2 while IgM and IgE lacks hinge region and instead have an additional constant domain.

Each heavy and light chains contains an amino (NH_2) terminal and a carboxylic (COOH) terminal. Variable region (V) containing about 110 amino acids is present at the amino terminal of a chain. The amino acid of the light (V_L) and heavy (V_H) chains varies greatly among antibodies of different specificity. It's the variable region of a heavy or light chain that is responsible for antigen binding. The remaining part of the chain excluding the variable region (V) is called as Constant region (C); the sequence of amino acids is relatively constant. The constant region of heavy and light chain is abbreviated as C_H and C_L respectively.

All the differences in specificity displayed by different antibodies towards antigens can be traced to differences in the amino acid sequences of the variable (V) regions. The area in the variable region that constitutes the antigen binding site of the immunoglobulin is called as complementarity determining regions (CDRs) of variable region. The remaining variable region of heavy and light chain shows less variation and are known as framework regions (FRs).

Deducing antibody structure

In the year 1950's and 1960's experiments by Rodney R. Porter (England) and Gerald M. Edelman (USA) elucidated the basic structure of the immunoglobulins and for there significant contribution; the two investigators shared a Nobel Prize in the year 1972.

Porter and Edelman first separated the gamma globulin of the serum designated as IgG and then subjected IgG to brief digestion with plant proteinase papain. The papain cleaves the IgG molecule in the hinge region between $\text{C}_\gamma 1$ and $\text{C}_\gamma 2$ domains to give two identical Fab fragments and one F_C fragment (Figure 4). It was noted that the Fab region is concerned with antigen binding site while F_C region mediates effector function such as placental transmission, complement fixation and monocytes binding. Fab fragment stands for "fragment, antigen binding"; each with a molecular weight of 45,000 Dalton while F_C stands for "fragment, crystallizable"; as it was found to crystallize during cold storage.

Another significant experiment was done by Alfred Nisonoff, in which the IgG molecule was subjected to enzyme pepsin digestion which generated two major fragments, one the $\text{F}(\text{ab}')_2$ fragment which broadly encompasses the two Fab fragment linked at the hinge region by disulfide bond. However the F_C region was not recovered from pepsin digestion because it has been digested into multiple fragments and could not be eluted.

The multichain structure of IgG given by Edelman was confirmed by Porter by subjecting it to mercaptoethanol reduction and alkylation, which irreversibly reduced and cleaved the disulfide bonds between the (H-H) chain and (H-L) chains. Thus chromatography of 150,000 molecular mass IgG molecule revealed that it is composed of two 50,000 MW heavy chains and two 25,000 MW light polypeptide chains (Figure 4).

From these experimental results, Porter and Edelman proposed a structure of IgG and suggested the basic structure of Immunoglobulins.

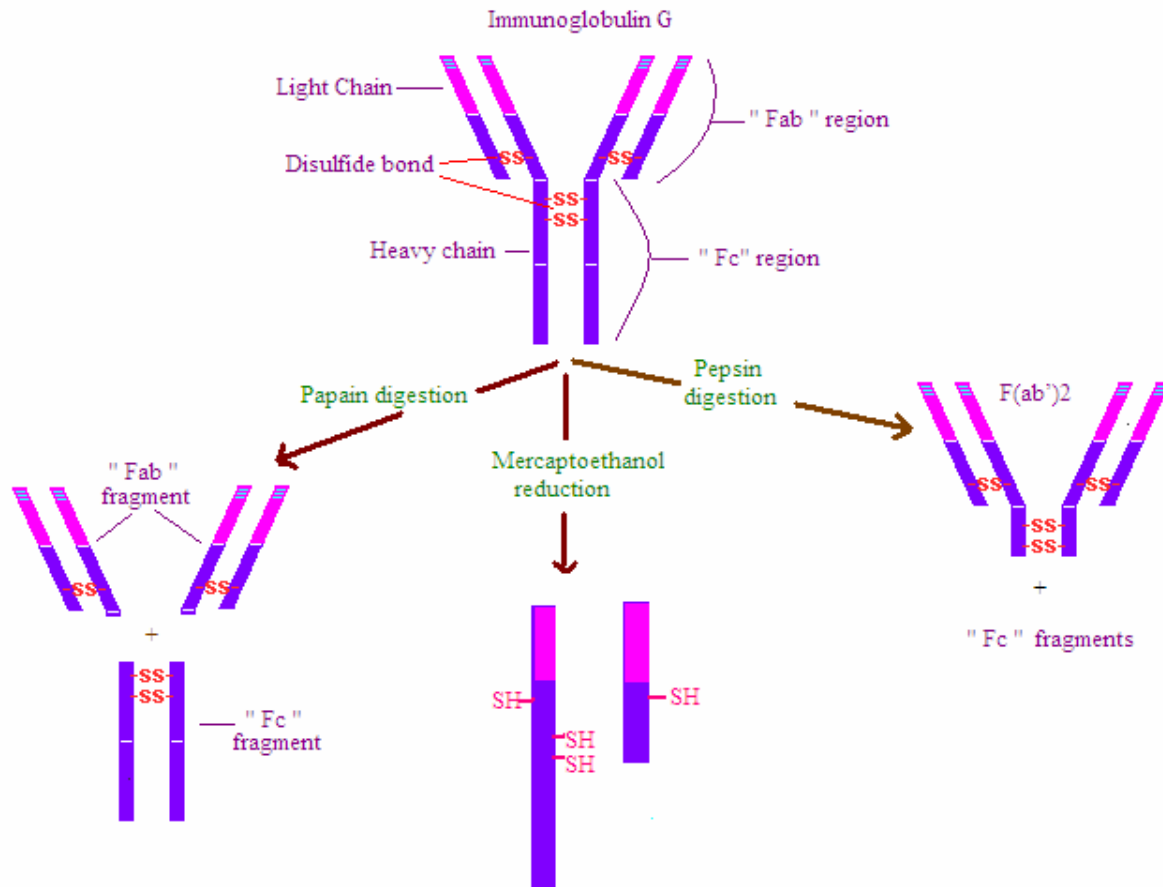


Figure 4 - Experimental methods followed to deduce the basic prototype antibody structure (IgG) by Porter and Edelman, and Alfred Nisonoff.

PRIMARY STRUCTURE OF IMMUNOGLOBULIN

The Immunoglobulins found in any serum sample are a complex mixture of molecules with antibody activity against a wide spectrum of epitopes hence they form a heterogeneous antibodies in the sample. Immunoglobulins are secreted by plasma cells mainly B cells. Occasionally a single plasma cell may become neoplastic, resulting in the development of cancerous plasma cells that produce a single, absolutely homogeneous monoclonal immunoglobulin product. In normal life cycle of lymphocytes, the plasma cells are end stage cells that secrete monoclonal antibodies for a few days and then die but in multiple myeloma (a cancer of antibody-producing plasma cells) as described above escapes the normal controls on their life span and proliferate continuously and divide in a unregulated manner. These cancerous plasma cells are called as myeloma cells. The antibodies produced by such cancerous cells are indistinguishable from the normal antibody molecule and are called myeloma proteins, denotes its originating cells. In most multiple myeloma patients, myeloma cells secretes large amount of light chain and is called as Bence-Jones protein. They were first discovered by scientist Bence and Jones in the urine of myeloma patients.

Light Chain Sequences

The amino acid sequence in the C-terminal (carboxyl group terminal) half of each light chain is found to be almost identical, hence called as constant light region (C_L). Each light chain

contains about 214 amino acids. While the sequences in the N-terminal (free amino group terminal) half are different in each light chain and called as variable (V_L) region. The constant light region (C_L) has two basic amino acid sequences; these are demarked that there are two light chain types i.e. Kappa (κ) and Lambda (λ).

In humans 60% of light chains are constituted by kappa (κ) and 40% are lambda (λ) while in mice 95% of light chains are kappa and only 5% are lambda. The amino acid sequence of lambda light chain shows minor difference.

Genes for λ - light chain in humans and mouse are found at chromosome 22 and 16 respectively while for κ - light chain at chromosome 2 and 6 respectively and for heavy chain they are found at chromosome 14 and 12 respectively in human and mouse.

Heavy Chain Sequences

Each heavy chain of IgG consists of about 445 amino acids. Analysis shows that the sequence of the 115 amino (N) terminal amino acids are different in each heavy chain and constitute a variable (V_H) heavy chain (Padlan E.A., 1994). The remaining 330 amino acids that constitute the constant (C_H) heavy chain region show five basic sequences corresponding to five different heavy chain constant regions.

Each of the five different heavy chains ($\mu, \delta, \gamma, \epsilon, \alpha$) forms five major types of Immunoglobulin Isotypes (Table 2). The heavy chains of a given antibody molecule determines the class of that antibody or Immunoglobulin: IgM (μ), IgD (δ), IgG (γ), IgE (ϵ), IgA (α).

Table 2 - Nomenclature and basic properties of five major classes of immunoglobulin.

Nomenclature and Symbol used	Previous Nomenclature	Heavy chain	Properties
Immunoglobulin E (IgE)	Reagin or IgND	ϵ	Binds to mast cells releasing histamine responsible for allergic reaction.
Immunoglobulin A (IgA)	γ A globulin or β_2 A-globulin	α	Present in tears, nasal mucus, breast milk, intestinal secretions.
Immunoglobulin D (IgD)	γ - SJ	δ	Present in B-cell plasma membranes.
Immunoglobulin M (IgM)	γ - macroglobulin or γ M globulin	μ	Present in B-cell plasma membrane; mediates initial immune response; activates complement components against bacterial infection.
Immunoglobulin G (IgG)	γ G globulin or 7S γ globulin	γ	Primary blood-borne soluble antibodies; crosses placenta (Passive immunization).

Hinge Region of Immunoglobulins

The Fab regions of IgG are mobile and flexible hence can swing freely around the center of the molecule as if they are hinged. The hinge region consist about 15 amino acids located between the C_{H1} and C_{H2} regions (Figure 5). The hinge region is present only in IgG, IgD, and IgA of the five major classes of immunoglobulins while absent in IgM and IgE. Although the IgE and IgM lacks the hinge region, so an additional C_{H2} / C_{H2} domains are present in

them at the same place in the heavy chain polypeptide chain as the hinge region in IgG, IgD, and IgA. The exact function of this extra domain in IgE and IgM has not been deciphered yet.

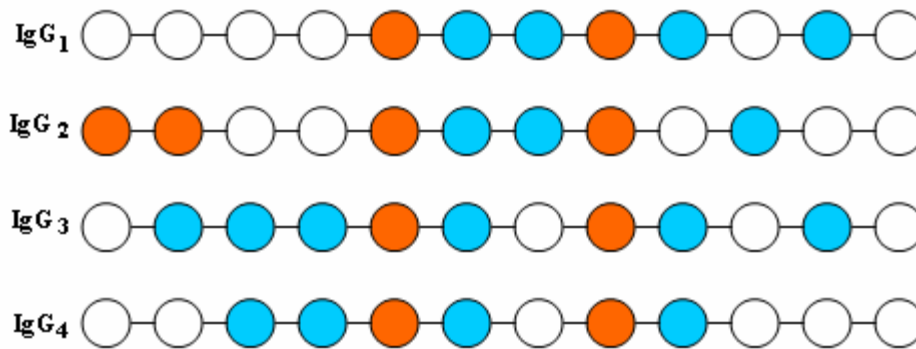


Figure 5. - Showing the hinge region amino acid sequence of human IgG subclasses IgG1, IgG2, IgG3 and IgG4 respectively. The blue circle denotes proline residues (that confers flexibility) while orange circle denotes cysteine residues (that forms interchain disulfide bridges).

The hinge region contains a large number of hydrophilic and proline amino acids. Proline and cysteine amino acids are present in most prominence at this region. The cysteine amino acids forms the interchain disulfide bonds between the two heavy chains in the immunoglobulin molecule while proline residues because of its configuration produces a 90° bend thus producing a universal joint around which the immunoglobulin chains can swing freely.

HUMAN IMMUNOGLOBULIN CLASSES, SUBCLASSES, TYPES AND SUBTYPES

Immunoglobulin classes (Isotypes)

The immunoglobulins are divided into 5 different classes, based on the differences in the amino acid sequences of the constant region of the heavy chains (C_H region). All the immunoglobulins within a given class will have very similar heavy chain constant regions. They are as follows:

- IgA - Alpha heavy chains (α)
- IgE - Epsilon heavy chains (ϵ)
- IgM - Mu heavy chains (μ)
- IgD - Delta heavy chains (δ)
- IgG - Gamma heavy chains (γ)

Immunoglobulin Subclasses

The classes of immunoglobulins can be further divided into subclasses based on small differences in the amino acid sequences of the constant region of the heavy chains (C_H region). Only IgG and IgA immunoglobulins have subclasses. All the immunoglobulins within a subclass will have very similar amino acid sequence at the heavy chain constant region. They are as follows:

IgG Subclasses

- IgG1 - Gamma 1 heavy chains
- IgG2 - Gamma 2 heavy chains
- IgG3 - Gamma 3 heavy chains
- IgG4 - Gamma 4 heavy chains

IgA Subclasses

- IgA1 - Alpha 1 heavy chains
- IgA2 - Alpha 2 heavy chains

Immunoglobulin Types

Immunoglobulins can also be classified by the type of light chain. These light chain types are based on differences in the amino acid sequence at the constant region of the light chain (C_L region). They are kappa light chains (κ) and lambda light chains (λ).

Immunoglobulin Subtypes

The light chains of immunoglobulins can also further be divided into subtypes based on small differences in the amino acid sequences in the constant region of the light chain (C_L region). The Lambda (λ) subtypes are $\lambda 1$, $\lambda 2$, $\lambda 3$, $\lambda 4$.

Presence of heterogeneity in immunoglobulins

Immunoglobulins are considered as a population of molecules in a heterogeneous state as they are composed of different classes and subclasses and each of which are further classified as different types and subtypes of light chains. And moreover different immunoglobulin molecules can have different antigen binding properties because of different V_H and V_L regions in them.

IMMUNOGLOBULIN FINE STRUCTURE

Immunoglobulin Domains

Studies have showed that both heavy and light chains contain Immunoglobulin domains. Each domain contains 110 amino acids; an intra-chain disulfide bond forms a loop of about 60 amino acids. Light chains contains: one variable domain (V_L) and one constant domain (C_L) while heavy chain contains one variable domain (V_H) and either three or four constant domain (C_H).

Each domain is folded into a characteristic immunoglobulin fold. This structure contains “sandwich” of two antiparallel β pleated sheets, which are connected by loops of various length. The two β pleated sheets within a fold are stabilized by many hydrogen bonds that connect the NH_2 group of one strand to $COOH$ group of the other strand (Figure 6). Further hydrophobic interaction between the two sheets and conserved disulfide bond makes the fold more stable.

In the intact IgG₁ immunoglobulin molecule (Padlan E.A., 1994) two heavy and light chains combine to form an IgG₁ immunoglobulin molecule. The heavy and light chains contains 110 amino acids units or domains.

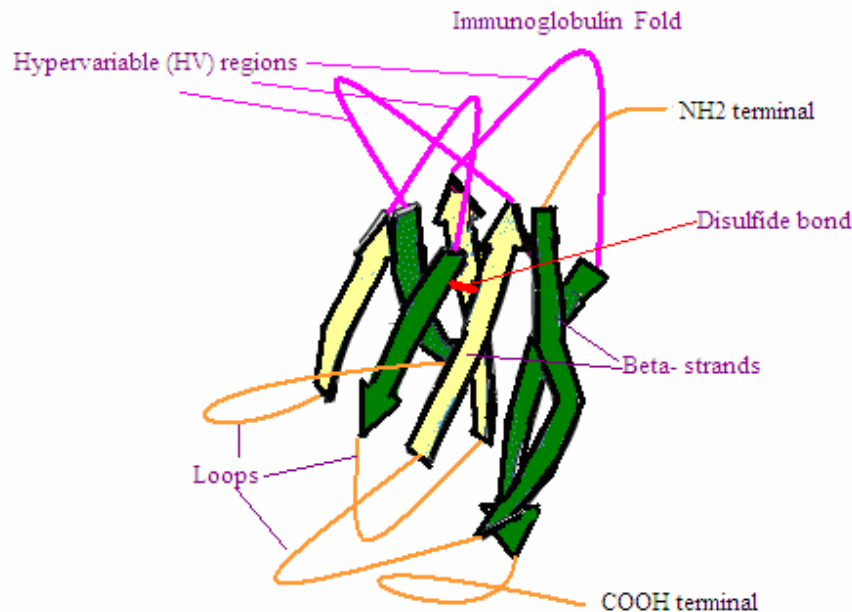


Figure 6- Diagrammatic representation of an intact variable region domain of an immunoglobulin. Each immunoglobulin domains of variable or constant region are folded into characteristic compact structures known as **immunoglobulin fold** (Ig fold). This fold consists of a “sandwich” of two antiparallel β pleated sheets, each of which are connected by loops. Variable region (V_H or V_L) contains three **hypervariable (HV) regions**, which forms the antigen binding sites of the antibody molecule.

Variable region domains fold

When the amino acids sequences of a large number of variable regions from both light and heavy chains are examined carefully, the sequences variability is concentrated in several hypervariable (HV) regions.

Both kappa (κ) and lambda (λ) light chains and heavy chains possess three hypervariable regions, constituting 15%-20% of variable region domain. Each hypervariable region is relatively short, consists of six to ten amino acids. The remaining part of the variable region of both heavy (V_H) and light (V_L) chain exhibits less variation and are called as framework regions (FR_S).

The hypervariable regions are also called complementarity determining regions (CDRs), as these regions are antigen binding sites of the antibody molecule and are complementary to the structure of the antigen epitope.

In the IgG₁ molecule each folds of variable region contains 9 β strands arranged in two sheets of 4 and 5 strands. The 5 stranded sheet is almost structurally similar to 3 stranded sheet of constant region but contains extra C' and C'' strands while the 4 stranded sheet contains A, B, E, and D strands. The disulfide bond between strand B and F stabilizes the domain fold.

Constant region domain fold

Each domains in the immunoglobulin structure has a similar structure of two β pleated antiparallel sheets tightly packed against each other. This conserved structure is also known as Immunoglobulin fold.

The IgG₁ molecule immunoglobulin fold of light constant (C_L) domain containing a 3-stranded sheet packed against a 4-stranded sheet. The two β sheets are stabilized by hydrogen bonding between the β strands of each sheets, by hydrophobic bonding between residues of opposite sheets in the interior and by the conserved disulfide bond between the sheets. The 3 stranded ones sheet contains C, F and G strand while A, B, E and D forms the second 5 stranded sheet.

Major effector function mediated by Immunoglobulin constant domain

As the variable region of the antibody is responsible for specific binding with antigen, the constant region of heavy chain is responsible for invoking effector response of the humoral immunity through interaction with other proteins,, lymphoid cells, basophils, eosinophils, mast cells etc. Few of the major effector responses are Opsonization, Activation of complement system, Antibody dependent cell mediated cytotoxicity (ADCC). The Fc region of antibody classes and subclasses (Table 4) show different mode of reactivity to complement, passive immunity and different proteins as well as different binding ability (Table 5) to lymphocytes and granulocytic cells.

Table 4 – Major properties of determined by Fc region of antibody classes and subclasses

Immunoglobulin	IgG1	IgG2	IgG3	IgG4	IgM	IgA	IgD	IgE
Ability to fix Complement	++	+	+++	-	+++	-	-	-
Placental transfer (Passive immunity)	+	+ or -	+	+	-	-	-	-
Reactivity with staphylococcal protein A	+	+	-	+	-	-	-	-

Table 5- Binding ability of different classes and subclasses of Immunoglobulins

Immunoglobulin class	IgG1	IgG2	IgG3	IgG4	IgM	IgA	IgD	IgE
Mononuclear cells	+	-	+	-	-	-	-	?
Neutrophils	+	-	+	+	-	+	-	-
Mast cells and Basophiles	-	-	-	?	-	-	-	+++
T and B lymphocytes	+	+	+	+	+ [#]	+ [#]	-	+
Platelets	+	+	+	+	-	-	-	?

+[#], restricted to subpopulations of these cells

Opsonization

It is the process which promotes the clearance of antigens by phagocytosis and opsonin are the molecules (like IgA, IgG and C3b, C4b components of complement system) that increase the process further.

Macrophages (mononuclear phagocytic cells present in tissue) and Neutrophils ((also known as polymorphonuclear leukocytes) are the most active phagocytic cells. These phagocytic cells contains Fc receptors (FcR) on their cell surface, which mediate the binding of the constant region of the antigen bound IgG molecules (Table 3). This crosslinking of Fc receptor and Fc region of antigen loaded antibody causes activation of signal transduction pathway resulting in phagocytosis of antibody-antigen complex and the pathogen is damaged to killed by enzymatic and oxidative stress processes. Phagocytosis of foreign antigen acts as stimulus for activation of macrophages and can be further stimulated by interferon gamma (IFN- γ), the most potent activators of macrophages secreted by activated T_H cells.

Table 3 - Functions related to IgG domains

IgG domains	Functions performed
V _H + V _L	Antigen binding
C γ 1	Complement (C4b) fragment binding
C γ 2	Complement (C1q) fixation
C γ 3	Binds to Fc receptor on macrophages and monocytes
C γ 4	Binds to Fc receptor on placental syncytiotrophoblast, neutrophils, K cells

Activation of Complement System

They form the major part of the humoral immune response. Most of the proteins and glycoproteins that constitutes the complement system is produced mainly by the liver hepatocytes. These forms about 5% of the globulin fraction in serum and circulate in functionally inactive state. They are also called as zymogens or proenzymes due to their ability to be active enzyme only after proteolytic cleavage of its inhibitory part. All the complement pathways begins with formation of antigen-antibody complex followed by sequential activation of enzyme cascade of the complement system finally forms a macromolecular structure called membrane attack complex (MAC). One of the active part C3b of the C3 component is a major opsonin. The C3b binding to non-specific site near the activation region of infected cells causes phagocytosis of the cells or antigen-antibody complex better.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

The crosslinking of antibody bound with tumor or virus infected cell to the Fc receptor of natural killer (NK) cells. The NK cells express an CD16 (Fc receptor) for constant region of the IgG molecule, and the virus infected target cell is destroyed by apoptosis. This process is called as Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC).

Structural features and biological properties of five major human immunoglobulin Isotype classes

Immunoglobulin G (IgG)

It is the immunoglobulin isotype found in highest concentration in serum, constitutes about 80% of the total serum immunoglobulins (Table 1). It plays the major role in antibody mediated defense mechanism like the transfer of IgG from mother to fetus (a type of passive

immunity, which is the acquisition of immunity by receipt of preformed antibodies rather than by active production of antibodies after exposure to antigen).

It has a relative molar mass of about 160,000 and relatively small in size. Due to its small size it can escape from blood vessels more easily than other immunoglobulins. IgG can opsonize, agglutinate, precipitate antigen and can also activate the classical pathway of complement activation.

There are four human IgG subclasses accorded by the differences in the gamma (γ) heavy chain sequences. They are IgG1, IgG2, IgG3, and IgG4 (Figure 7). The four subclass of IgG shows some specific properties, they are as follows:-

- IgG1, IgG3, IgG4 readily cross the placenta and provides passive immunization to the fetus.
- IgG3, IgG1, IgG2 can activate complement system and are arranged to their decreasing effectiveness.
- IgG1 and IgG3 mediate opsonization by binding with high affinity to F_C receptors on phagocytic cells and mediate antibacterial defenses.

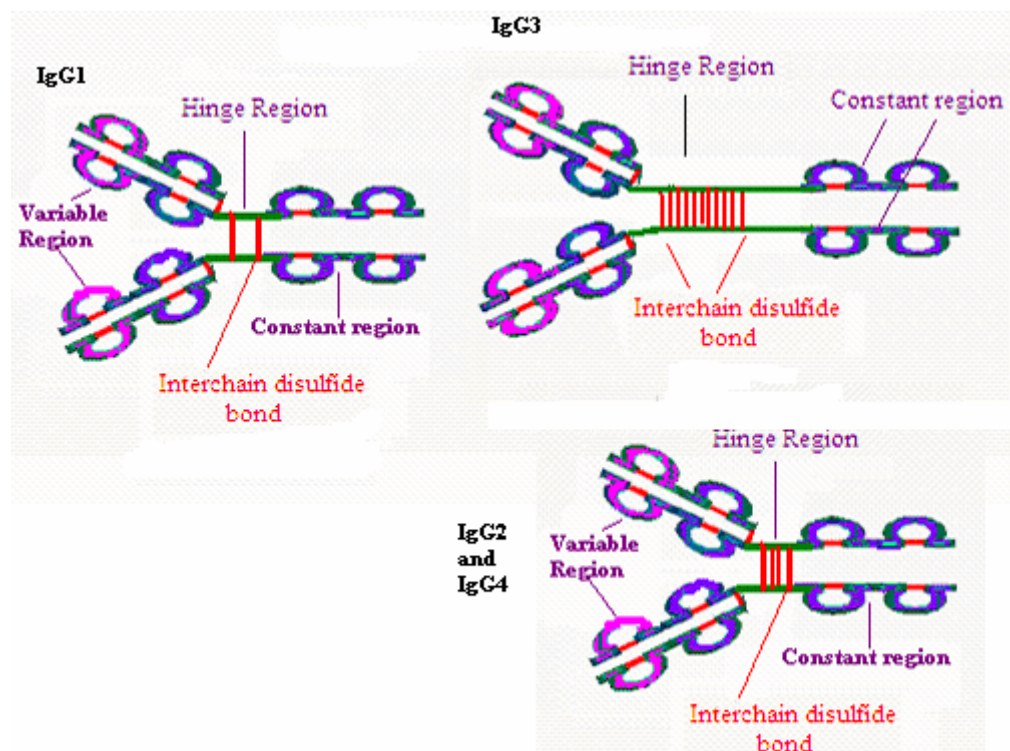


Figure 7.- General structures of the four subclasses of human IgG, which differ in number of interchain disulfide bonds (like IgG3 has 11 disulfide bonds).

Immunoglobulin M (IgM)

Immunoglobulin M accounts for 5 % to 10% of the total serum immunoglobulins. Structurally, IgM is formed by five basic monomeric IgM (molecular weight of 180,000 of each monomer). IgM is secreted by plasma cells as a pentamer in which five monomer units are held together by disulfide bonds that link their carboxyl terminal heavy chain domains ($C\mu 4 / C\mu 4$) and ($C\mu 3 / C\mu 3$) domains (Figure 8). Each pentamer contains an additional F_C linked polypeptide called as J chain (Joining chain), which is disulfide bonded to carboxyl

terminal cysteine residue of two of the ten heavy (μ) chains. The molecular mass of the pentamer is 900,000 and each monomer subunit possesses two antigen binding sites so the valency of IgM for antigen would be 10 but in practical, this valency is more commonly found to be 5 because of steric hindrance between antigen molecules.

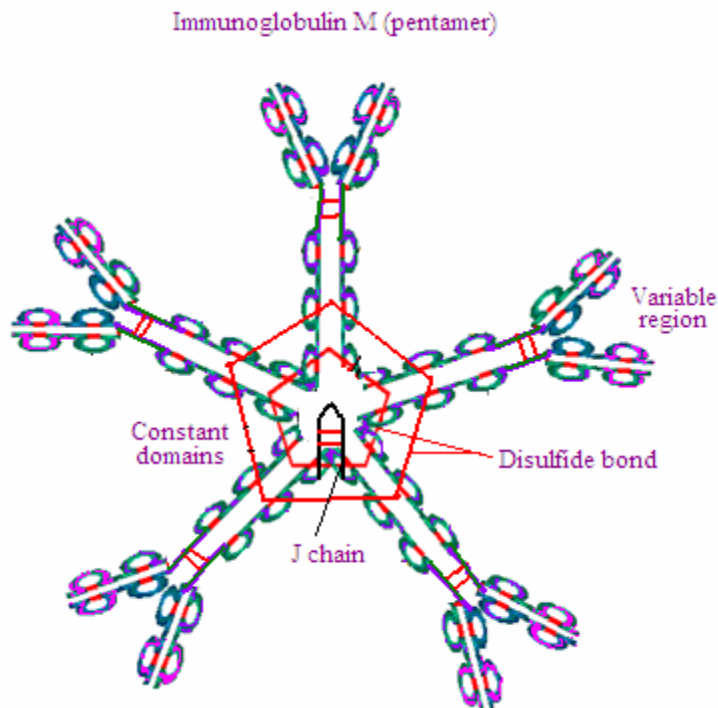


Figure 8- General structure of secreted IgM, which is made up of five monomer units. Each pentamer contains an additional Fc linked polypeptide called J (joining) chain. IgM monomer lacks hinge region.

IgM is the major immunoglobulin isotype produced in a primary immune response. It is also produced during a secondary response, but this is commonly masked by very large quantity production of IgG molecules. In humans about 32 mg/Kg of IgG is produced daily and only 2.2 mg/Kg of IgM is produced.

IgM is the first immunoglobulin class produced in a primary response to an antigen. Because of its high valency, pentameric IgM is more efficient than other isotypes in binding multidimensional antigen as viral particle and red blood cells. The large size of pentameric IgM makes them confined to blood vascular system and are therefore of little importance in conferring protection in tissue fluids and body secretions. IgM is more efficient than IgG at activating the complement cascade, opsonization, virus neutralization and agglutination.

IgM molecules in monomeric form function as antigen receptors on B cells. This membrane bound IgM (mIgM) differs from the secreted IgM (sIgM) form by presence of longer C-terminal C_H4 region along with hydrophobic residues that form the trans membrane domain and enable the mIgM to interact with cell membrane lipids.

Immunoglobulin A (IgA)

Monomeric IgA has a relative molar mass of about 160,000 and sedimentation coefficient of 6.8 S or 7S, but it is normally secreted in dimer form. IgA constitutes only 10% to 15% of the total serum immunoglobulins. Secretory IgA (sIgA) is of critical importance in body

protection like against invading micro organisms; since it is the major immunoglobulin in the intestinal respiratory and urogenital tracts, tears, milk, and saliva.

Each IgA molecule has a typical four chain structure consisting of paired kappa (κ) or lambda (λ) light chains and two alpha (α) heavy chains. In the dimer IgA, the molecule are joined by a J chain (joining chain) linked to the F_C region of monomers. The J chain helps to form the dimer form (Figure 9). The IgA molecule exists as monomer in the serum but dimer, trimer and tetramer are sometimes observed while the secretory IgA exists as dimer. IgA has two subclasses IgA₁ and IgA₂ which differs in the heavy chain subtypes with a half life of about 6 days (Table 1).

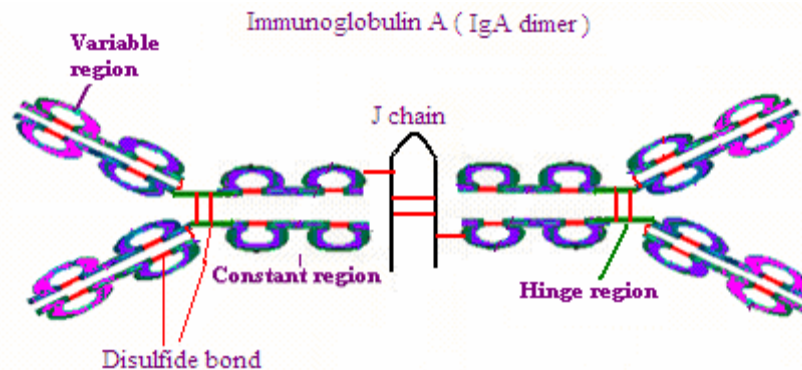


Figure 9- General structure of IgA dimmer. Secretory IgA consists of dimmer formed during transport through mucous membrane epithelial cells. In humans, most serum IgA molecules exists as monomers, even though some dimers, trimers and even tetramers are sometimes present.

The mucous membrane lining of the digestive, respiratory and urogenital systems are safe guarded by specialized lymphoid tissue known as mucosal **associated lymphoid tissue (MALT)**. The major function of MALT is production of large quantity of antibody-producing plasma cells than those produced in spleen and bone marrow combined. IgA of external secretions is called as secretory IgA (sIgA), which consists of a dimer or tetramer, a J chain polypeptide and a polypeptide chain called secretory component. When foreign antigens that invaded the body; are transported across the mucous membrane by M cells (specialized cells to carry out transport of antigens across mucous membrane) and activates the B cells in lymphoid follicles. The activated B cells differentiate into IgA producing plasma cells after leaving the follicles and the secretory IgA is formed during transport through mucous membrane epithelial cells. The IgA secreting plasma cells are concentrated along the mucous membrane surfaces like jejunum of the small intestine. The plasma cells producing IgA migrate to subepithelial tissue. Dimeric IgA binds to poly-Ig receptor expressed on basolateral surface of most mucosal epithelia. After polymeric IgA binds to receptor, and the receptor-IgA is transported across the epithelial membrane to the lumen. The poly-Ig receptor is enzymatically cleaved, releasing the secretory component bound to the dimeric IgA into the mucous secretion, where they can interact with the antigens.

The secretory IgA acts as a major effector at mucous membrane surface. As the bacterial and viral antigens enter the body through mucous membrane, the dimeric or tetrameric secretory IgA binds with them and prevent their attachment to the mucosal cells thus inhibiting the bacterial or viral infection. The antibody-antigen complex is further cleared by ciliated epithelial cells of intestinal or respiratory tracts.

Breast milk contains secretory IgA that help protect the new born against infection. Secretory IgA has been proved as essential line of defense against salmonella, vibrio cholerae, polio virus, influenza etc.

Immunoglobulin E (IgE)

It is composed of paired kappa (κ) or lambda (λ) light chains and two epsilon (ϵ) heavy chains. Each heavy chain contains four constant region domains (Figure 10). IgE has a molar weight of about 200,000 Dalton and 8S sedimentation coefficient. IgE is present in extremely low average serum concentration (0.3 $\mu\text{g/ml}$). IgE mediates the immediate hyper-sensitivity reactions and is largely responsible for immunity to invading parasitic worms. Due to its importance to mediate Type I hypersensitivity reaction, it is called as reaginic antibody.

IgE is unique as its F_C region binds strongly to receptors on mast cells and basophils inducing degranulation and causing release of substances that mediates allergic manifestations. IgE has the shortest half-life of all immunoglobulins (2 to 3 days) and is readily denatured by mild heat treatment.

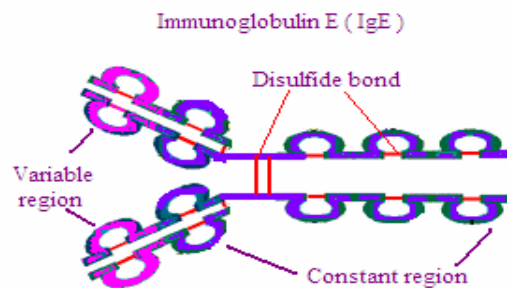


Figure 10- General structure of IgE molecule. It lacks the hinge region.

IgE also provides defence mechanism against parasites. As the IgE binds to Fc receptors on basophils and mast cells. The allergen cross-linkage of Fc receptor bound IgE molecule on tissue mast cells causes degranulation with release of few pharmacologically active mediator particles along with the granules. These mediators help in formation of various types of cells required for antiparasitic attack.

Immunoglobulin D (IgD)

IgD is a trace immunoglobulin accounting less than 1% of the total serum immunoglobulin (Figure 11). IgD molecule consists of two heavy delta (δ) heavy chain and two kappa (κ) or lambda (λ) light chains. Its relative molar mass is about 180,000 (7S sedimentation coefficient).

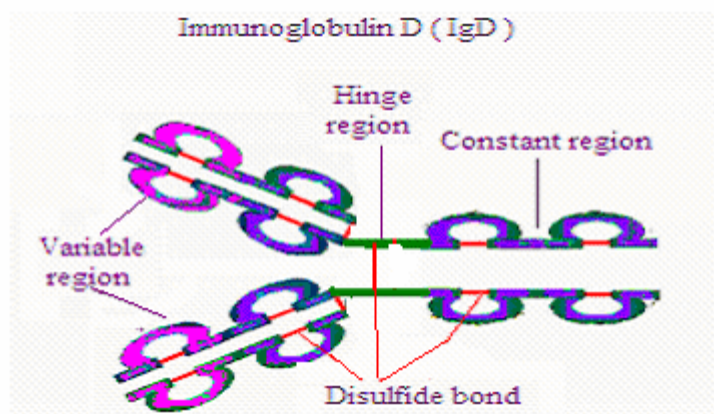


Figure 11- General structure of IgD monomer.

IgD structure has a single disulfide bond between the delta (δ) chains and a high content of carbohydrate distributed in multiple oligosaccharide units. IgD together with IgM is the major membrane bound immunoglobulins expressed by mature B cells. IgD is usually absent from serum, but it is found in very low concentration in plasma.

The major class and subclasses of five immunoglobulins have varying degree of reactivity towards different lymphocytes and platelets (Table 7).

ANTIGENIC DETERMINANTS ON IMMUNOGLOBULINS (IMMUNOGLOBULIN VARIANTS)

Due to the glycoprotein composition of antibodies, they can themselves function as potent immunogens. Such anti-Ig antibodies are useful for study of B cells development and humoral immune response. The antigenic determinants (or epitopes) of immunoglobulins are classified into three major groups i.e. Isotype, Allotype and Idiotypic; depending on the characteristic position on the immunoglobulin molecule (Figure 12).

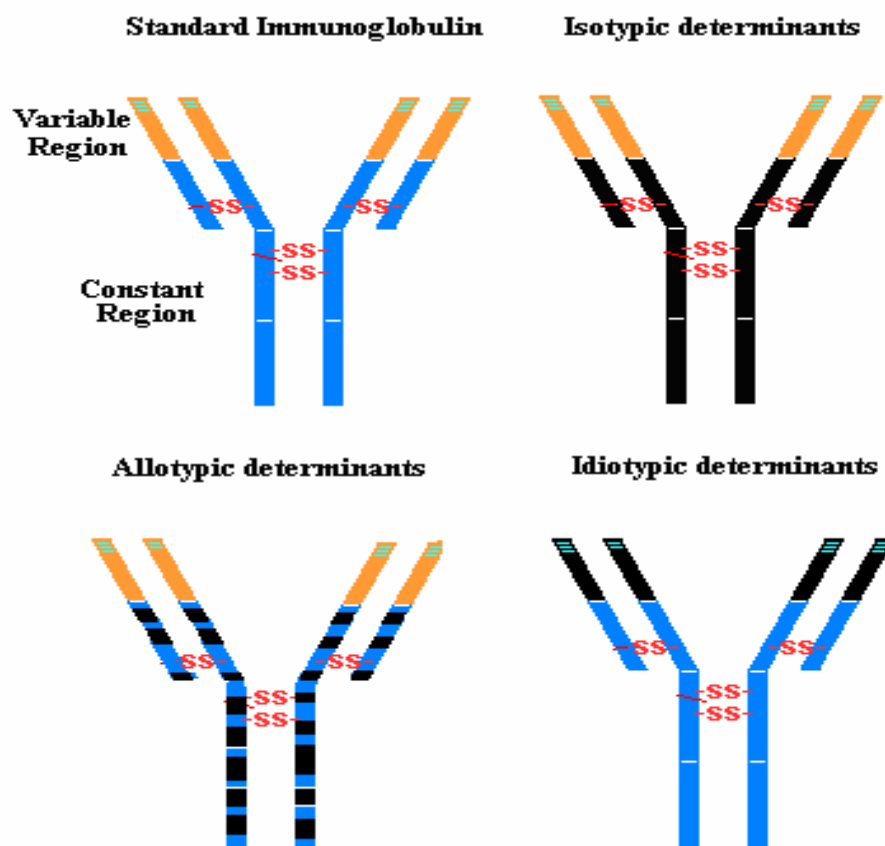


Figure 12- Antigenic determinants (black regions) of immunoglobulins. Isotypic determinants are constant region determinants, Allotypic determinants are subtle amino acid differences in the isotypic genes, and Idiotypic determinants are variable region determinants.

Isotype

The genes for isotypic variants are present in all healthy members of species. These are represented by constant region determinants that additively define each heavy chain and light chain within a species. Different species express different isotypes, hence when an antibody of a species is injected to a different species organism; the injected antibody is recognized as

foreign body or antigen and thus inducing an antibody response in the recipient to the isotypic determinants on the foreign antibody. In research fields, these anti-isotype antibodies are used to determine the class or subclass of serum antibody produced during immune response in immunological test or experiments.

Allotype

This refers to the genetic variation within a species involving different alleles at given locus in the isotype genes. These alleles encode subtle amino acid differences within the constant region. Allotypes occur mainly as variants of heavy chain constant regions; these allotypes occur in some but not all members of a species. In humans allotype have been characterized for four IgG subclasses, one IgA subclass and kappa (κ) light chain. The gamma chain allotypes are called as Gm markers. Almost 25 different Gm markers have been designated and are classified by the class and subclass followed by allele number like G2m (23), G4m (4a) etc. IgA2 subclass has allotypes A2m (1) and A2m (2) while the kappa light chain has κ m (1), κ m (2) and κ m (3).

Antibodies to allotypic determinants can be produced by injecting antibodies from one member of a species to another member of same species. Antibodies to allotypic determinants can arise during blood transfusion.

Idiotypic

Marked differences in the variable region (V_H or V_L) particularly in the highly variable segments known as hypervariable regions (HV), produce idiotypes. Idiotypic variation occurs in variable region only and idiotypes are specific to each antibody molecule.

Each antibody will present multiple idiotopes which may be actual antigen binding site or may comprise variable region sequences outside the antigen binding sites. The sum of individual idiotopes of the antibody is called as the idioform of the antibody. The anti-idioform antibodies have minimal variation in amino acid sequence at isotype and allotypes level, hence the idiotypic differences can be studied and observed.

DIFFERENT ANTIBODY RESPONSE FOLLOWING ANTIGEN IMMUNIZATION

Immunity is the state of protection from infectious diseases. It has both nonspecific and specific components. The nonspecific is called as the innate immunity and comprises of anatomic barrier, physiological barriers, phagocytic barriers and inflammatory barriers. This set of host defense mechanism is not specific to a particular pathogen.

The second level of defense is known as adaptive immunity which increases in strength and effectiveness with each encounter. It displays a high degree of antigenic specificity, diversity, immunologic memory and self/nonself recognition characteristic features. The foreign agent (antigen) is recognized in a specific manner and the immune system acquires memory towards it.

The first encounter with an antigen is known as the primary response. Re-encounter with the same antigen causes a secondary response that is more rapid and powerful (Figure 13). This heightened state of immune response towards the same antigen is due to the presence of memory cells (memory B cells); which have a longer life span than naive cells while plasma cells or effector cells produce antibody in secreted form and helps to neutralize antigens during primary response.

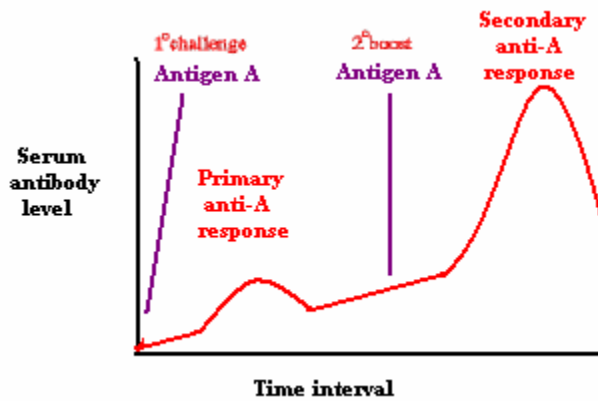


Figure 13- Differences in the primary and secondary response to injected antigen. When an animal is injected with antigen, it produces a primary serum antibody response of low magnitude and short duration while in second immunization with same antigen; a heightened and longer duration secondary immune response is obtained.

IMMUNOGLOBULIN SUPERFAMILY

The immunoglobulin fine structure contains immunoglobulin fold domains. The structure of the immunoglobulin molecule is determined by primary, secondary, tertiary, and quaternary organization of the polypeptide. The amino acid sequence of both variable and constant region of both heavy and light chain constitutes the primary structure, folding of the extended polypeptide chain back and forth to form an antiparallel β pleated sheet forms the secondary structure. The sheets are then folded into compact globular domains to form tertiary structure, each of domains are contiguously attached by continuation of the polypeptide chain that lie outside the β pleated sheets. In the final hierarch globular domains interact in the quaternary structure to form functional domains that enable the immunoglobulin molecule to bind to antigens and perform its designated effector functions.

X-ray crystallography analyses have revealed that immunoglobulin domains are folded into a compact, specific structure known as immunoglobulin fold. Each of these immunoglobulin folds contains two antiparallel β pleated sheets “sandwiched” and held together by hydrogen bonds between the two polypeptide chains.

A large number of proteins molecules have shown to bear one or more regions homologous to an immunoglobulin domain; that are characteristic of an immunoglobulin structure. All the protein molecules having such domains are grouped under Immunoglobulin Superfamily. The similarity between various membrane proteins suggests that these proteins have a common evolutionary ancestry. There genes have evolved independently and do not share genetic linkage or function.

The following proteins including immunoglobulins are representative members of the Immunoglobulin superfamily (Figure 14):

- Ig- α / Ig β heterodimer of B-cell receptor
- Poly- Ig receptor of secretory IgA molecule
- T-cell receptor
- T-cell accessory proteins, including CD2, CD4, CD8, CD28, and the γ , δ , and ϵ chains of CD3

- Class I and Class II MHC molecule
- β_2 - microglobulin of Class I MHC molecule
- Cell adhesion molecules like LFA-3, ICAM-1, ICAM-2, and VCAM-1
- Platelet-derived growth factor

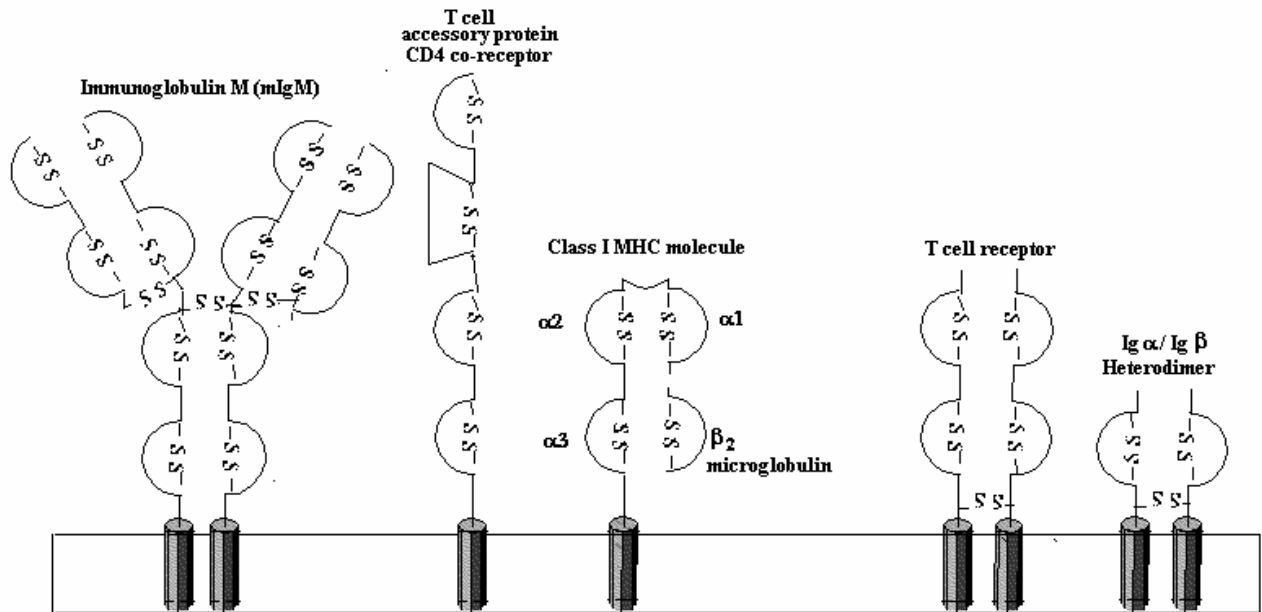


Figure 14 – Showing few members of the immunoglobulin superfamily. The molecule under this family contains the characteristic immunoglobulin fold domain structures having antiparallel β pleated sheets.

MONOCLONAL ANTIBODIES

The term **monoclonal antibodies** is used to denote immunoglobulin molecules that are derived from a single clone and recognize a single specific clone. Almost all the antigens possess multiple epitopes and henceforth they induce a variety of B-cell clones, each of which recognizes a particular epitope. The serum containing the antibodies against various epitopes (called as **polyclonal antibodies**) is known as heterogeneous mixture.

In research and therapeutic fields, the monoclonal antibodies are used, as they are specific for particular epitope. The analysis studies have shown that polyclonal antibodies have reduced efficacy for various *invitro* studies than *in vivo* studies hence the monoclonal antibodies are used that specific and effective *invitro* also. Georges Kohler and Cesar Milstein in 1975 formulated a technology for preparing monoclonal antibody. In this technique they fused a myeloma cancerous plasma cell with a normal, activated antibody producing B cell and this hybrid is called as Hybridoma. This hybridoma contained cumulative property of both B cell and myeloma cell. For there such significant work Kohler and Milstein were awarded with Nobel Prize in 1984.

Hybridoma formation and Selection by HAT medium

The primed B cells (from animals that have been immunized with specific antigen) capable of producing antibodies specific against epitope were fused with myeloma (cancerous plasma) cells by polyethylene glycol. Hybridomas have both the immortal property of cancerous cell as well as genetic information for synthesis of the specific antibody against specific epitope

of interest from the primed B cells. In the fusion process, a complex mixture of unfused and fused cells is produced. Three different combination of fused cells are produced, they are: unwanted (B cell x B cell), unwanted (Myeloma cell x Myeloma cell), and desired hybrid (B cell x Myeloma cell).

Now the next process is to specific selection of hybridomas to allow their survival and growth. One common method used for selection is to grow all the fused cells in HAT medium which contains hypoxanthine, aminopterin and thymidine.

HAT selection is based the fact that the mammalian cells are capable of synthesis of nucleotide by two different pathways: the *de novo* pathway and the salvage pathway. HAT medium contains aminopterin that blocks the *de novo* pathway while hypoxanthine and thymidine allow growth by salvage pathway. Hence unfused or fused cells that lack either hypoxanthine-guanine phosphoribosyl transferase (HGPRT) enzyme or thymidine kinase (TK) enzyme will die in HAT medium because then they cannot utilize the salvage pathway (Figure 15).

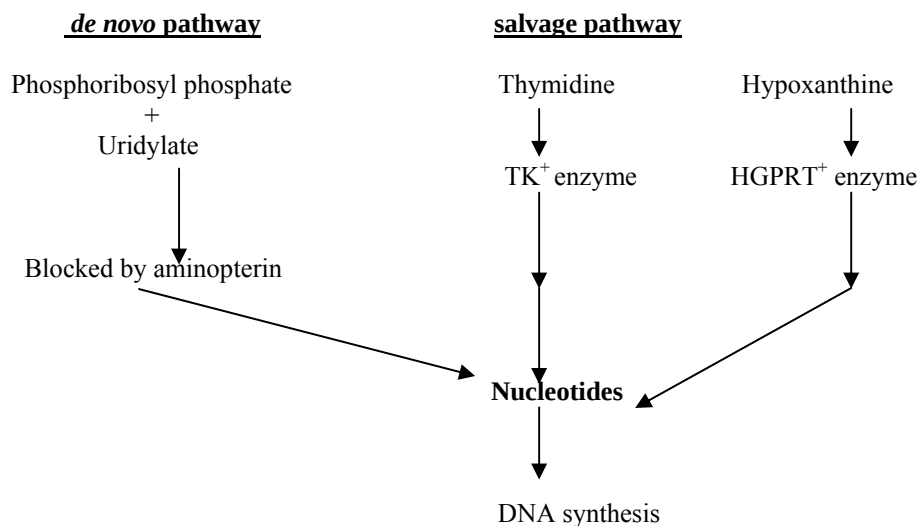


Figure 15- Overview of *de novo* and salvage pathways. Aminopterin (act as an analog of dihydrofolic acid) blocks the *de novo* pathway inhibiting purine synthesis. The salvage pathway utilizes hypoxanthine and thymidine for nucleotide synthesis in presence of HGPRT enzyme (Hypoxanthine-guanine phosphoribosyl transferase).

In hybridoma technology, fused (myeloma cell x myeloma cell) or unfused myeloma cell lack the HGPRTase enzyme and therefore get deselected and killed in HAT medium. Although unfused B cells are able to survive in HAT medium as they contain the HGPRTase enzyme but these cells don't live for extended period of time as they lack the immortal property and die out invitro (Figure 16). The hybridoma (B cell x myeloma cell) can only survive because it has both HGPRTase enzyme as well as immortal growth. As the desired hybridoma survives in HAT medium, it uses the hypoxanthine and thymidine to synthesis its nucleotides and maintain its viability.

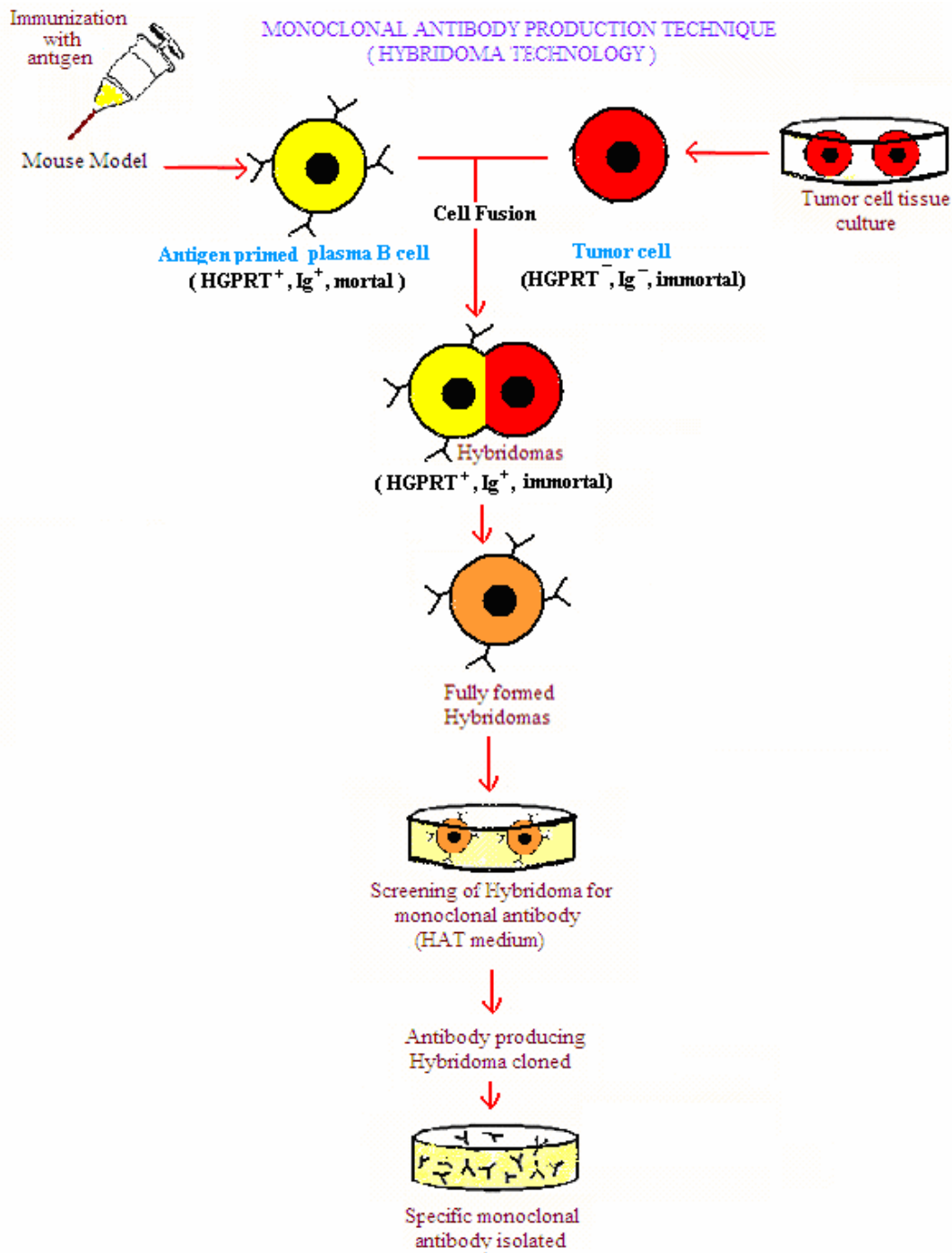


Figure 16- Outline of the basic method to prepare monoclonal antibody. The monoclonal antibodies are derived from a single plasma cell (B cell) that is specific against a particular epitope on a complex antigen.

Production and Screening of Monoclonal antibodies

A monoclonal antibody is derived from a single plasma cell and is specific for one epitope on a complex heterogeneous antigen. The most common screening method for the desired antigenic specificity is: ELISA (Enzyme-Linked Immunosorbent Assay) and RIA (Radioimmunoassay).

After the identification of specific hybridoma, they are recloned to produce desired monoclonal antibody as they can be grown in tissue culture flask or in peritoneal cavity of histocompatible mice where the monoclonal antibodies are secreted in higher concentration into the ascites fluid and then can be separated and purified by chromatography techniques. Monoclonal antibodies secreted into the medium in case of cloning in tissue culture flask is very low concentration about 1 to 20 µg/ml while in the mice ascites fluid is about 1 to 10 mg/ml. New high density technique have been developed for large quantity production.

Few recent research reports have stated that multidrug-resistant variant of Sp2/0 mouse myeloma cell line SpEBr-5 has been used for making mouse hybridoma producing monoclonal antibodies. The resulting hybridoma cell line 1F7 can produce a high level of monoclonal antibody production and karyotype containing all normal mouse chromosomes. Studies on these adriamycin (ADR) - resistant 1F7 hybridoma cell lines have suggested that the higher level of specific immunoglobulins is due to the increased level of Igγ_{2b} heavy chain mRNA (M. B. Melixetian, M. A. Pavlenko et. al., 2003).

Recently a new human myeloma cell line (Karpas 707H) have been developed for hybridoma and monoclonal antibody production (Karpas A, Dremucheva A. and Czepulkowski B.H., 2001). This human myeloma cell line (Karpas 707H) is HAT (hypoxanthine – aminopterin – thymidine) sensitive and ouabain-resistant and contains unique genetic markers. This Karpas 707H myeloma cell line can easily be fused with ouabain-sensitive Epstein–Barr virus (EBV)-transformed cells or blood lymphocytes, hybridizing to produce stable hybrids which continuously secrete high quantities of human monoclonal immunoglobulins. The major advantage of these derived hybrids is that they do not lose the property of immunoglobulin secretion over many months of continuous growth. Thus such myeloma cell lines would enable scientist to immortalization of human antibody-producing B cells in vitro and monoclonal antibodies can be further used in diagnostic purposes.

Clinical uses of Monoclonal antibodies

The monoclonal antibodies have proved to be a major tool for research and therapeutic field. They have numerous applications and are an important key to scientific world.

- Used as diagnostic reagents for detecting pregnancy, various pathogenic diseases, matching histocompatibility antigens, blood grouping.
- Used in research field for studies on antigen-antibody interaction and used in techniques like Immunoprecipitation, Immunofluorescence, Flow cytometry, ELISA, RIA, Immunoelectron microscopy.
- Radiolabeled antibodies are used *in vivo* for detecting or locating tumor antigens, imaging techniques for various types of cancer. Monoclonal antibodies against breast cancer labeled with iodine-131 have been used in patients to detect the metastasis of tumor to regional lymph nodes.
- Tumor specific monoclonal antibodies coupled with lethal toxins called as Immunotoxins are potentially valuable therapeutic reagent. The toxins generally used to prepare the immunotoxins include *Shigella toxin*, diphtheria toxin, ricin etc and have shown to have lethal action against the diseased part. Immunotoxins consists of inhibitory toxin chain and the tumor antigen specific monoclonal antibody, hence the attached antibody specifically delivers the toxin to the tumor cells thus causing death of the infected target cell by inhibiting protein synthesis or other function that are caused by the toxin chain.

- Recombinant vaccines for deadly diseases like cancer, AIDS, malaria etc. are being developed using the monoclonal antibodies.

ABZYMES (CATALYTIC MONOCLONAL ANTIBODY)

An abzymes (word derived from antibody and enzyme) or catmab (word derived from catalytic monoclonal antibody), is a monoclonal antibody with catalytic activity. The binding of antibody to antigen and enzyme to substrate involves weak, noncovalent interaction with high specificity and affinity. In most cases, antibodies tightly bind the antigen, but do not specifically alter its chemical nature while the enzymes does. The “transition state theory”, states that enzymes catalyze a reaction by stabilizing the chemical intermediate known as transition state, a molecule intermediate between substrates and products. Production of such transition state geometry is energetically unfavorable in the absence of enzyme but in its presence the activation energy of the transition state is lowered and the reaction is pushed through its transition state to form product.

Abzymes are generally artificial constructs, but they are sometimes found in normal humans (anti-vasoactive intestinal peptide auto antibodies) and in patients with autoimmune disease like systemic lupus erythematosus (SLE or lupus), where they can bind and hydrolyze DNA. The transition-state analogs when injected into an animal, they act as antigen (hapten) to elicit antibody production. Such antibody developed to a stable molecule which is similar to an unstable intermediate of different potentially unrelated reaction will enzymatically bind to stabilize the transition-state molecule, thus the net resultant is catalyzation of the reaction. Abzymes have developed as an important and potential tool in biotechnology like to perform specific actions on DNA.

In year 1986, Peter Schultz and Richard Lerner generated abzymes that catalyze ester hydrolysis, i.e. the breakage of an ester bond by addition of water molecule. They demonstrated the hydrolysis reaction of p-nitrobenzoate involves the nucleophilic attack of the oxygen atom of water molecule on the carbonyl atom in p-nitrobenzoate to produce tetrahedral geometry transition state. Chemical scientist have reproduced analog of the proposed transition state intermediate by Schultz and Lerner. The rates of reactions catalyzed by abzymes (measured by K_M and V_{max} values) are up to a million-fold greater than the same uncatalyzed reactions. In few exceptions some catalytic antibodies have also not yet approached the rates of reactions catalyzed by natural enzymes. But the milestone experiments by Schultz and Lerner, however have led production of over 100 different abzymes that catalyze a wide range and variety of reactions.

GENETICALLY ENGINEERED ANTIBODIES

Chimeric antibody

It is formed by genetically engineered process of fusion of parts of a mouse antibody with parts of a human antibody. In general the chimeric antibodies contain approximately 33% mouse protein and 67% human protein and are majorly developed to reduce the HAMA (Human Anti-mouse antibody) response elicited by mouse antibodies. But these chimeric antibodies can induce a HACA response (Human Anti-Chimeric Antibodies) similar to HAMA response in the human body and thus reduces the chances to act as an therapeutic tool.

Humanized antibody

It is also a genetically engineered antibody that is formed by fusion of mouse part from a murine antibody onto the human antibody. These humanized antibodies consists of only 5-

10% mouse part while about 90-95% human and are being engineered to bypass the HAMA and HACA responses as seen in case of chimeric antibodies.

“Fully human” or “Human” Antibody

Recent research studies have developed “fully human” antibody that are derived from transgenic mice which carry the set of human immunoglobulin genes while “human” antibodies are derived from human cells. All the three; “fully human”, “human”, and “humanized” antibodies are of clinical importance as they invoke the immune response less and have a better safety profile as compared to other genetically engineered antibodies.

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