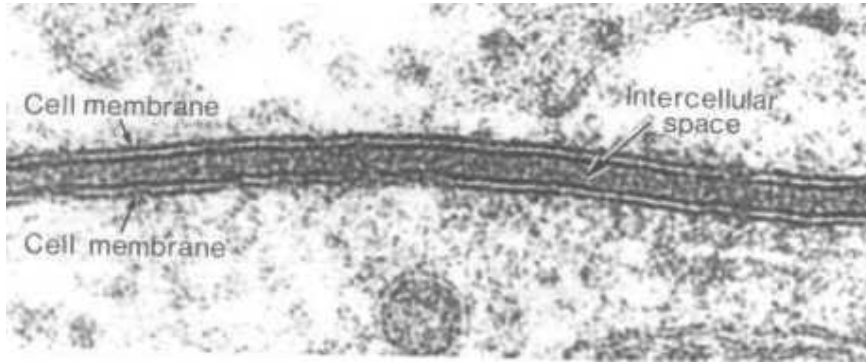


INTRODUCTION TO BLOOD GROUP SEROLOGY

CELL LAYER.....



Membrane Structure

The cell is highly organized with many functional units or organelles. Most of these units are limited by one or more membranes. To perform the function of the organelle, the membrane is specialized in that it contains specific proteins and lipid components that enable it to perform its unique roles for that cell or organelle. In essence membranes are essential for the integrity and function of the cell.

Membrane components may:

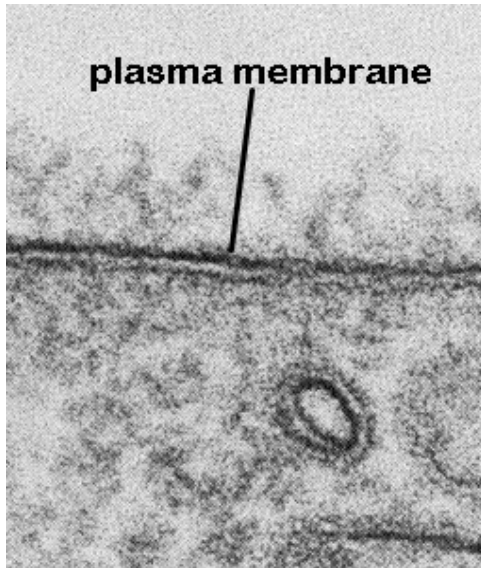
- be protective
- regulate transport in and out of cell or subcellular domain
- allow selective receptivity and signal transduction by providing transmembrane **receptors** that bind signaling molecules
- allow cell recognition
- provide anchoring sites for **cytoskeletal filaments** or components of the extracellular matrix. This allows the cell to maintain its shape and perhaps move to distant sites.
- help compartmentalize subcellular domains or microdomains
- provide a stable site for the binding and catalysis of enzymes.
- regulate the fusion of the membrane with other membranes in the cell via specialized **junctions**)
- provide a passageway across the membrane for certain molecules, such as in **gap junctions**.
- allow directed cell or organelle motility

Membrane Structure and Function

Introduction :

Membranes are vital because they separate the cell from the outside world. They also separate compartments inside the cell to protect important processes and events.

Cellular membranes have diverse functions in the different regions and organelles of a cell. However, at the electron microscopic level, they share a common structure following routine preparative steps. The above figure shows the typical "Unit" membrane which resembles a railroad track with two dense lines separated by a clear space. This figure actually shows two adjacent plasma membranes, both of which have the "unit membrane" structure. (Above figure taken from Bloom and Fawcett, *A Textbook of Histology*, Chapman and Hall, N.Y., 12th edition, 1994, Figure 1-2.)



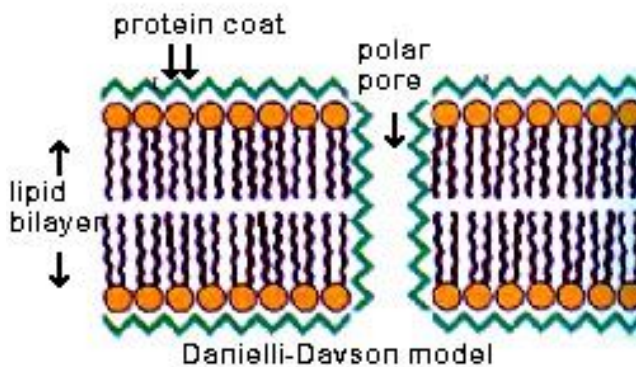
How did early cell biologists deduce membrane structure from electron microscopic images and the knowledge that membranes were lipoprotein complexes?

Note the material projecting from the plasma membrane both inside and outside of the cell. What do you think this is? Also, outside of the cell is in the upper half of photo. Can you find a vesicle with a unit membrane structure?

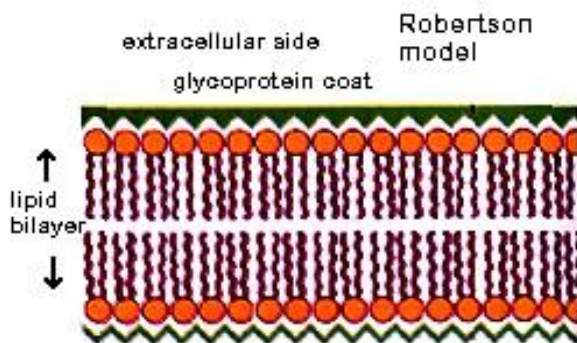
A historical perspective

In the early 1930's-40's, Danielli and Davson studied triglyceride lipid bilayers over a water surface. They found that they arranged themselves with the polar heads facing outward. However, they always formed droplets (oil in water) and the surface tension was much higher than that of cells. However, if you added

proteins, the surface tension was reduced and the membranes flattened out.



Here is a diagram of their early model of the cell membrane.



What membrane functions might be allowed by this model?

In the 1950's Robertson noted the structure of membranes seen in the above electron micrographs. He saw no spaces for pores in the electron micrographs. He hypothesized that the railroad track appearance came from the binding of osmium tetroxide to proteins and polar groups of lipids.

What is missing in Robertson's model?

In 1966, Lenard and Singer noted that over 30% of membrane proteins were twisted into an alpha helix. This made it likely that there were

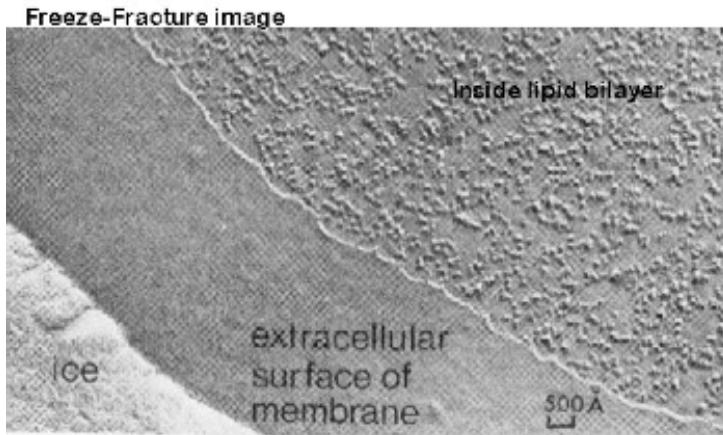
many spherical proteins.

Could one build a Davson-Danielli or Robertson model with spherical proteins? What would happen to the size of the membrane?

Furthermore, what would happen if you unfolded the proteins, exposing non-polar (hydrophobic amino acid side chains) groups to the aqueous environment?

What kind of energy would the cell have to expend to keep proteins flattened in this state?

Singer studied phospholipids bilayers and found that they can form a flattened surface on water, with no requirement for a protein coat. The turning point in the modeling came with the advent of freeze fracture techniques. This figure shows the inside of a membrane and the "bumps, grooves, ridges". These were later found to be proteins.

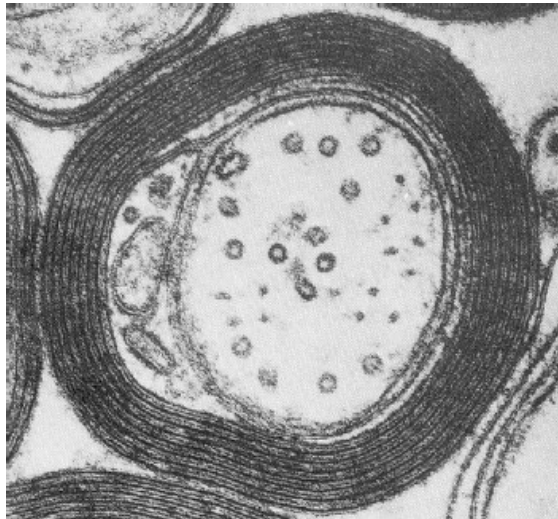


What conclusions could you draw about membrane structure from the photo to the left?

Basic Membrane Architecture

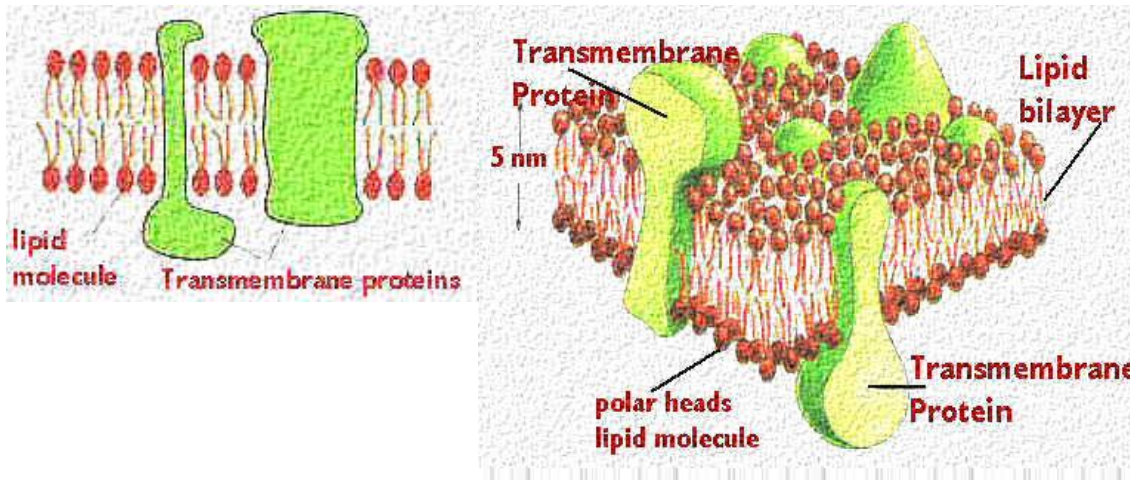
The purpose of this presentation will be to show how membranes are studied by the cell biologist. First, we will look at basic **unit membrane architecture**. All membranes contain proteins and lipid. However, the proportion of each varies depending on the membrane. For example:

- Myelin, which insulates nerve fibers, contains only 18% protein and 76% lipid. An electron micrograph of myelin is to the right.
- Mitochondrial inner membrane contains 76% protein and only 24% lipid.



- Plasma membranes of human red blood cells and mouse liver contain nearly equal amounts of proteins (44, 49% respectively) and lipids (43, 52% respectively).

Considering what you already know about these cells or organelles, what would be the significance of these different proportions of lipids and proteins?



As we said above, membrane architecture is that of a **lipid bilayer**. The lipids are amphipathic in that they have **hydrophilic polar heads** pointing out and the **hydrophobic portion** forming the core. Proteins are embedded in the bilayer. They may pass through the

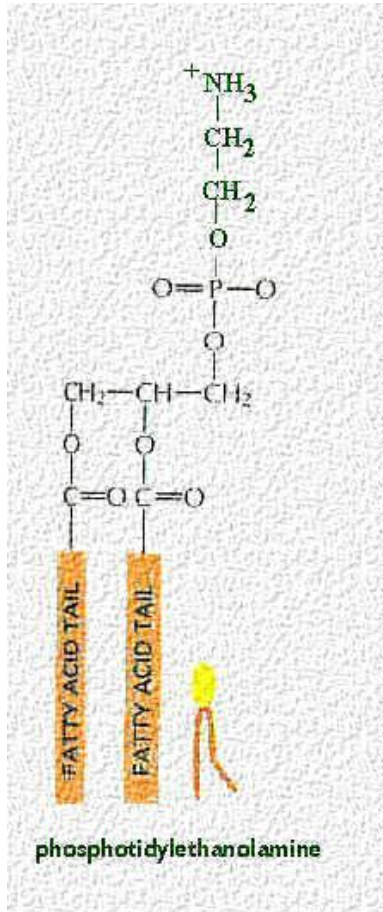
bilayer (as **transmembrane proteins**), or they may be inserted at the cytoplasmic or exterior face.

A cross section of the bilayer is seen in this figure. As we will see in more detail below, the lipid molecules have a **globular (polar) head** and a straight region (**non-polar**). Each row of lipids is a leaflet. Therefore, the plasma membrane consists of two leaflets with the non-polar regions pointing inward.

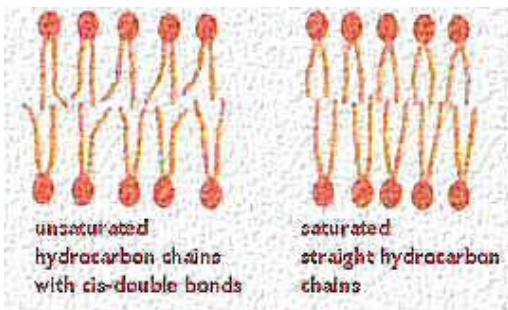
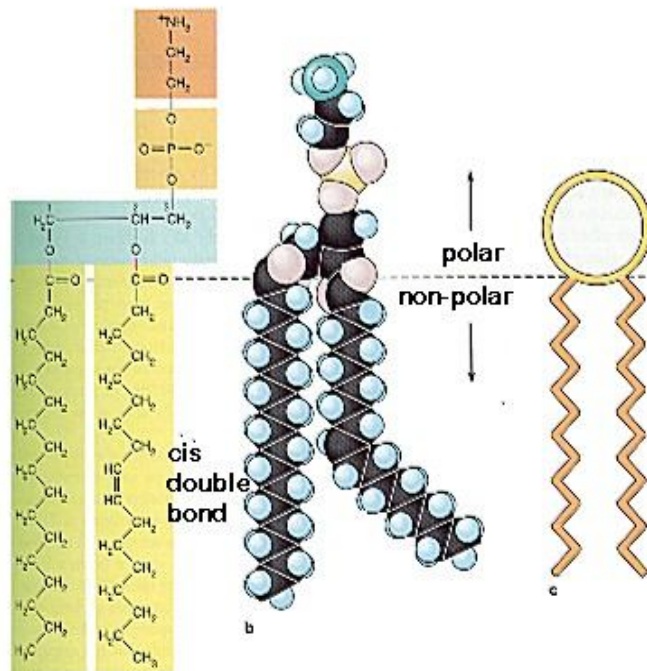
Membrane Phospholipids

Lets take apart the membrane and examine each of its components.

One of the principal types of lipid in the membrane include the **phospholipids**. These have a polar head group and two hydrocarbon tails. An example of a phospholipid is shown in this figure (right). The top region beginning with the NH₃ is the polar group. It is connected by glycerol to two fatty acid tails. One of the tails is a straight chain fatty acid (saturated). The other has a kink in the tail because of a cis double bond (unsaturated). This kink influences packing and movement in the lateral plane of the membrane. Figure to the left is modified from *Alberts et al. Molecular Biology of the Cell, Garland Publishing, N.Y., 1994, Third Edition, Figure 10-10*. The figure below is from *Wolfe S.L., Molecular and Cellular Biology*

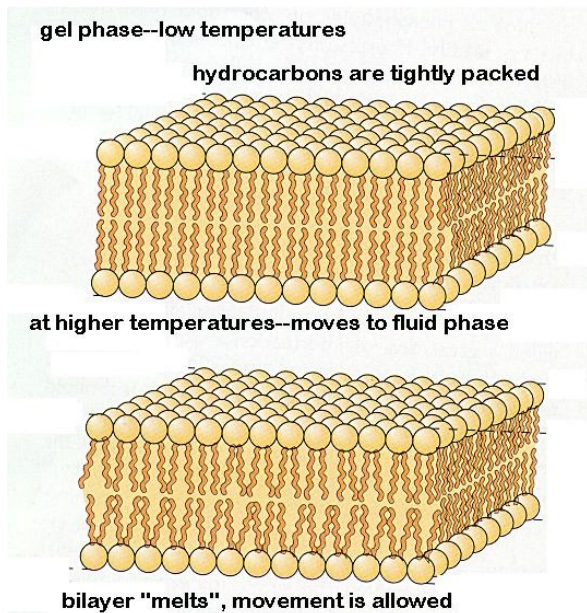


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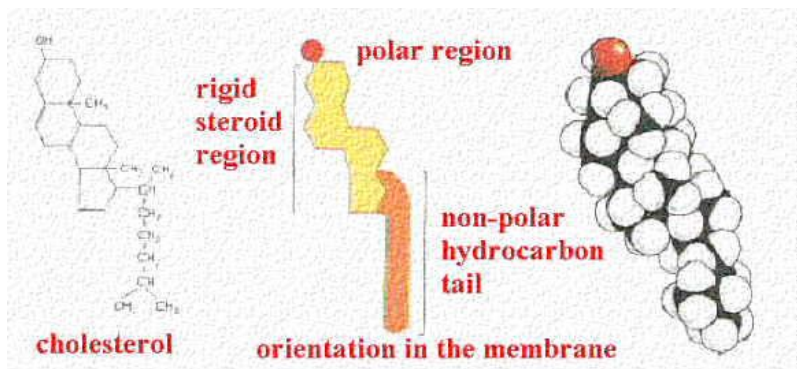
The figure in this paragraph shows how the phospholipids pack together in the two leaflets in the membrane. The presence of the cis double bond makes prevents tight packing and makes the bilayer difficult to freeze.

The lipid bilayer gives the membranes its fluid characteristics. The following cartoon shows the effect of temperature on the packing of the hydrocarbons. Note that at low temperatures, the bilayer is in a gel state and tightly packed. At higher (body) temperatures, the bilayer actually "melts" and the interior is fluid allowing the lipid molecules to move around, rotate, exchange places. This also allows movement of other components of the membrane. The figure below is from *Wolfe S.L., Molecular and Cellular Biology, Wadsworth Publishing Company, 1993*.

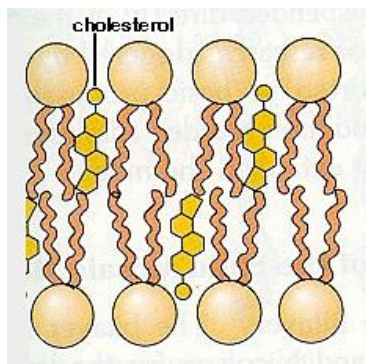


Membrane Cholesterol

Another type of lipid in the membrane is **cholesterol**. The amount of cholesterol may vary with the type of membrane. Plasma membranes have nearly one cholesterol per phospholipid molecule. Other membranes (like those around bacteria) have no cholesterol. The following figure shows the steroid structure of cholesterol. The non-polar and polar regions are also illustrated in b) (Figure modified from *Alberts et al. Molecular Biology of the Cell, Garland Publishing, N.Y., 1994, Third Edition, Figure 10-8.*



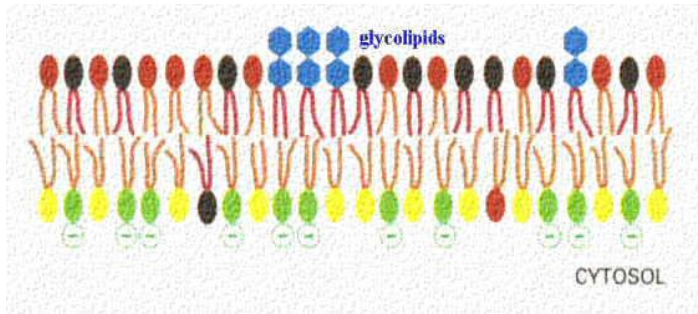
The cholesterol molecule inserts itself in the membrane with the same orientation as the phospholipid molecules. The figures show phospholipid molecules with a cholesterol molecule in between. Note that the polar head of the cholesterol is aligned with the polar head of the phospholipids. Figure modified from *Alberts et al. Molecular Biology of the Cell, Garland Publishing, N.Y., 1994, Third Edition, Figure 10-9;* or *Wolfe S.L., Molecular and Cellular Biology, Wadsworth Publishing Company, 1993 (figure below).*



Cholesterol molecules have several functions in the membrane:

- They immobilize the first few hydrocarbon groups of the phospholipid molecules. This makes the lipid bilayer less deformable and decreases its permeability to small water-soluble molecules. Without cholesterol (such as in a bacterium) a cell would need a cell wall.
- Cholesterol prevents crystallization of hydrocarbons and phase shifts in the membrane.

Membrane Glycolipids

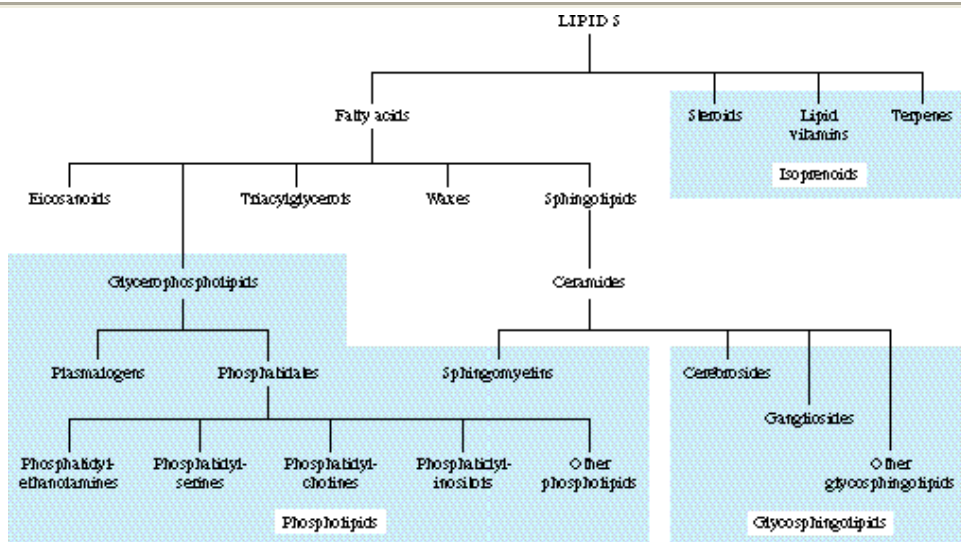


Glycolipids are also a constituent of membranes. In this figure, they are shown as blue sugar groups projecting into the extracellular space. They may microaggregate in the membrane. These components of the membrane may be protective, insulators, and sites of receptor binding. Among the molecules bound by glycosphingolipids include cell poisons such as cholera and tetanus toxins. The lower figure shows the chemical structure of two examples of glycosphingolipids.

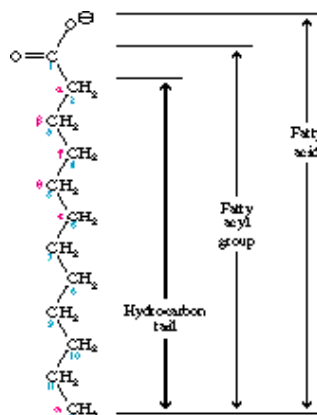
Formation of "Microdomains"

Sphingolipids and cholesterol work together to help cluster proteins in a region called a "microdomain". They function as "rafts" or platforms for the attachment of proteins as membranes are moved around the cell and also during signal transduction.

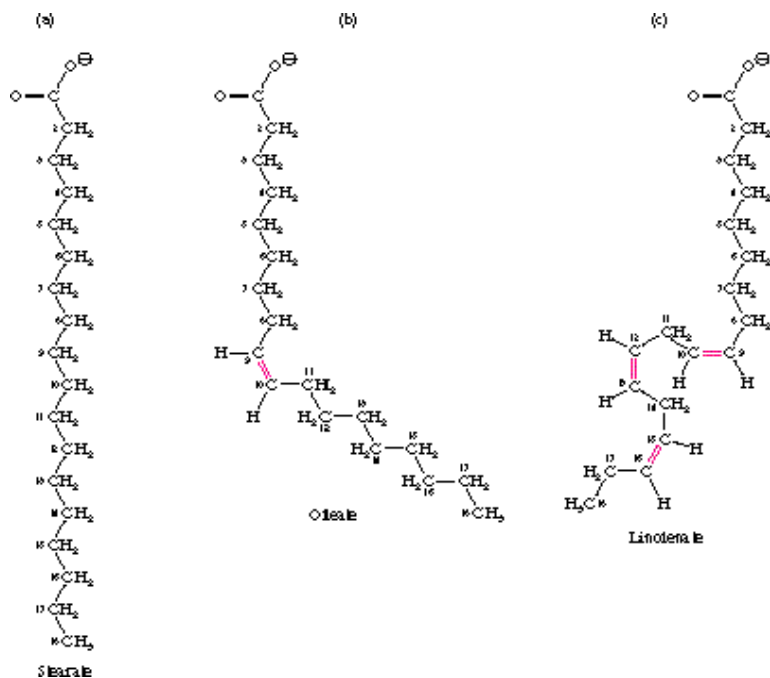
LIPID STRUCTURES



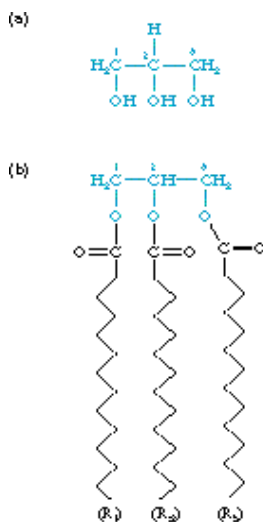
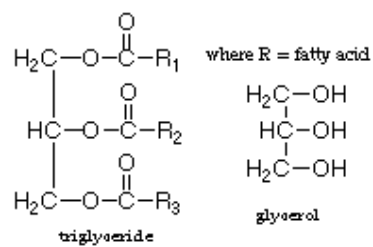
Structure and Nomenclature of Fatty Acids



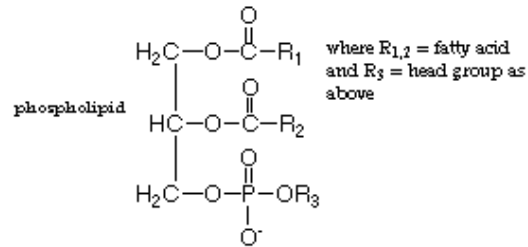
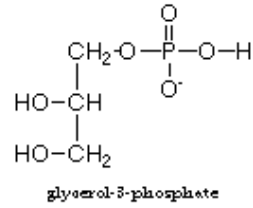
Structure of 3 C₁₈ Fatty Acids



Triacylglycerol



Glycerophospholipids or phosphoglycerides



a) Glycerol 3-Phosphate and Phosphatidate

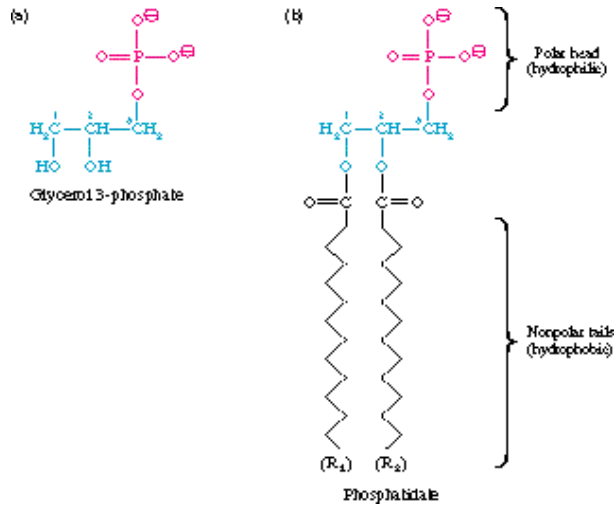
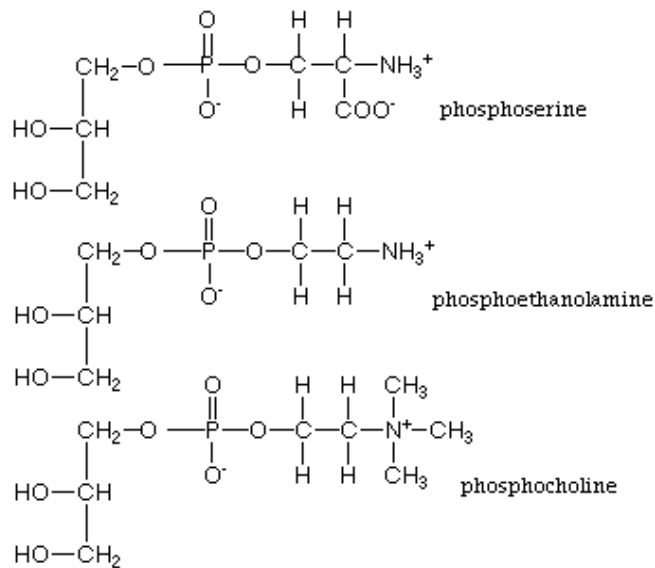


Table 10-2 Some common types of glycerophospholipids

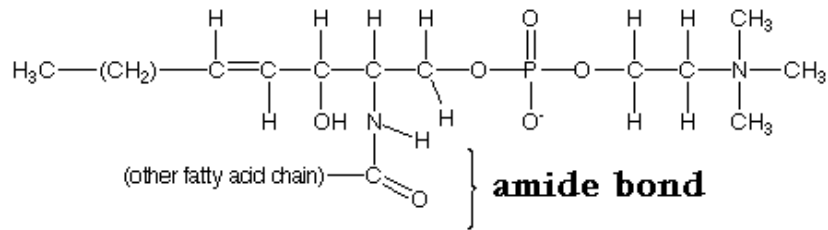
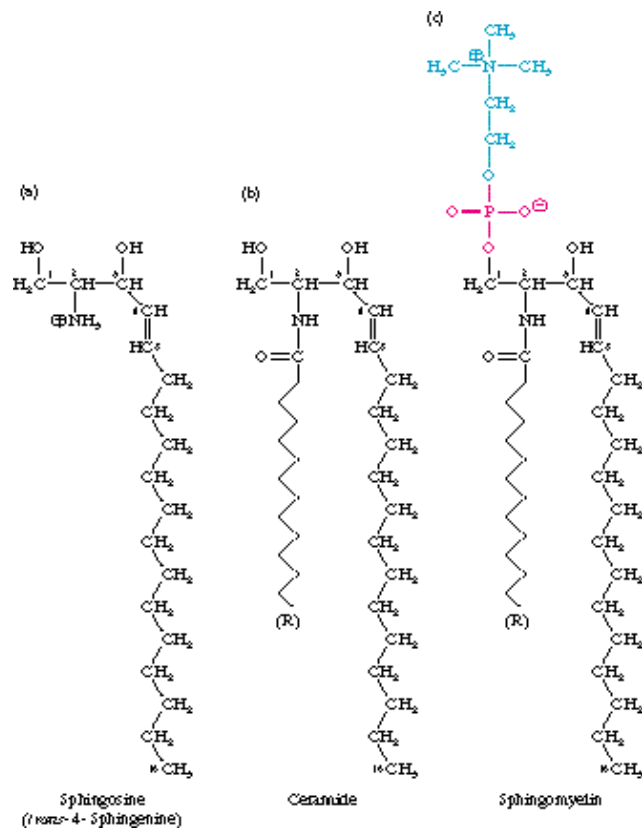
X = rest of polar head

Precursor of X (HO—X)	Formula of —O—X	Name of resulting glycerophospholipid
Water	—O—H	Phosphatidate
Choline	—O—CH ₂ CH ₂ N [⊕] (CH ₃) ₃	Phosphatidylcholine
Ethanolamine	—O—CH ₂ CH ₂ NH ₃ [⊕]	Phosphatidylethanolamine
Serine	—O—CH ₂ —CH(NH ₃ [⊕])COO [⊖]	Phosphatidylserine
Glycerol	—O—CH ₂ CH(OH)—CH ₂ OH	Phosphatidylglycerol
Phosphatidylglycerol	—O—CH ₂ CH(OH)—CH ₂ —O—P(=O)(O [⊖])—O—CH ₂ —CH(OH)—CH ₂ —O—C(=O)—R ₅	Diphosphatidylglycerol (Cardiolipin)
myo-Inositol		Phosphatidylinositol



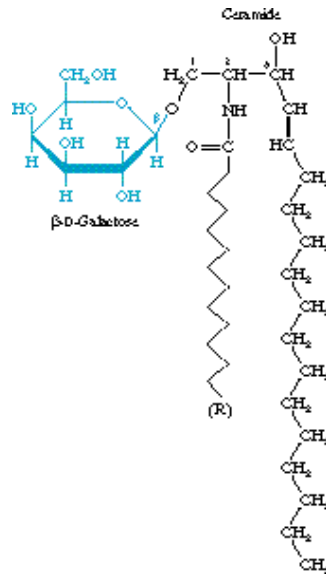
Sphingolipids

a) Sphingosine, b) ceramide, and c) Sphingomydin

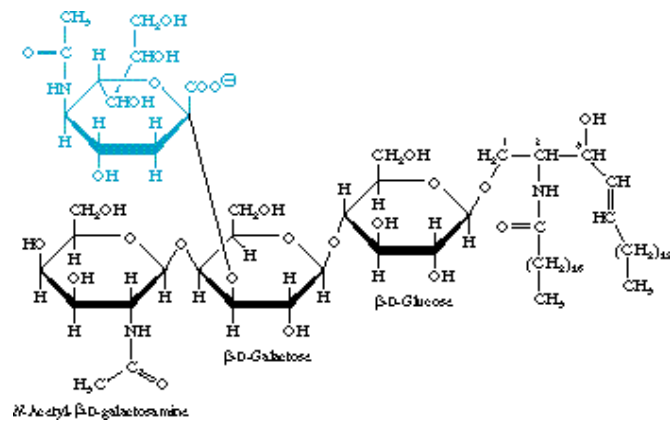


sphingomyelin

Galactocerebroside

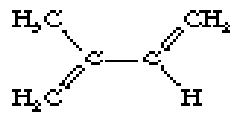


Ganglioside G_{M2}



Isoprenoids (based on 5-carbon isoprene)

Isoprene Unit



Membrane Proteins

As you study different organelles, you will learn about important membrane proteins that function for that particular organelle. **Transmembrane proteins** are amphipathic, in that they have hydrophobic and hydrophilic regions that are oriented in the same regions in the **lipid bilayer**. Another name for them is "**integral proteins**". Other types of proteins may be linked only at the cytoplasmic surface (by attachment to a fatty acid chain), or at the external cell surface, attached by a oligosaccharide. Or, these non-transmembrane proteins may be bound to other membrane proteins. Collectively these are called "**peripheral membrane proteins**". Proteins inserted once through the membrane are called "single-pass transmembrane proteins." Those that pass through several times are called "multipass transmembrane proteins". They form loops outside the membrane.

Glycoproteins are the proteins covalently attached to carbohydrates such as glucose, galactose, lactose, fucose, sialic acid, N-acetylglucosamine, N-acetylgalactosamine, etc. Glycolipids are carbohydrate-attached lipids. Their role is to provide energy and also serve as markers for cellular recognition.

The antigens which determine blood types belong to glycoproteins and glycolipids. The antigens which determine blood types belong to glycoproteins and glycolipids. There are three types of blood-group antigens: O, A, and B. They differ only slightly in the composition of carbohydrates.

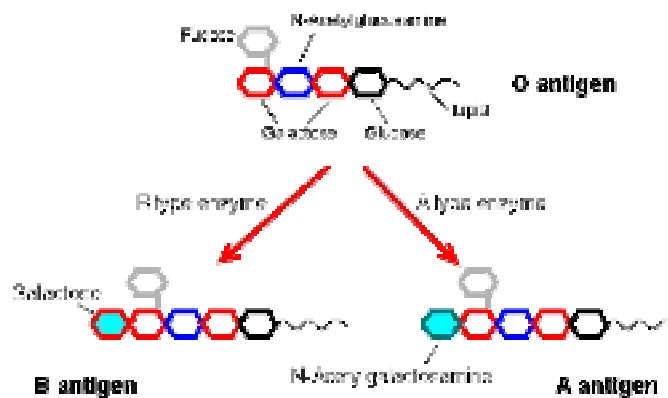


Figure. Blood-group antigens.

All humans contain enzymes which catalyze the synthesis of the O antigen. Humans with A-type blood also contain an additional enzyme (called A-type enzyme here) which adds N-Acetylgalactosamine to the O antigen. Humans with B-type blood contain another enzyme (called B-type enzyme here) which adds Galactose to the O antigen. Humans with AB-type blood contain both A-type and B-type enzymes while humans with O-type blood lack both types of enzymes.

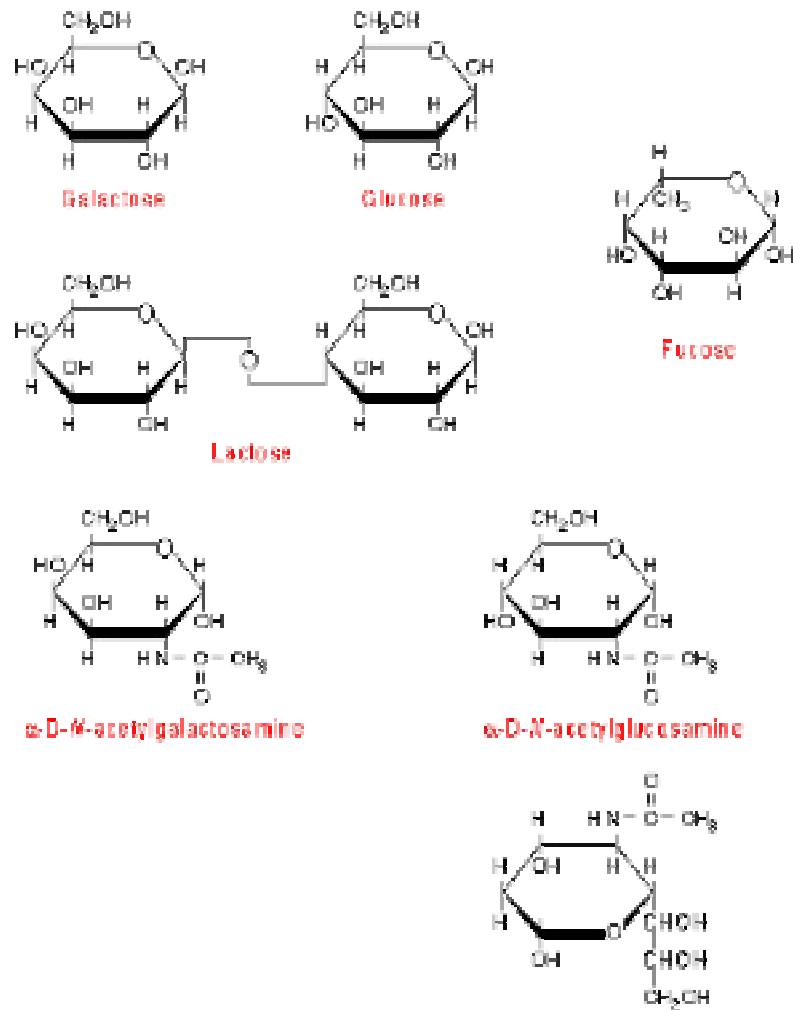
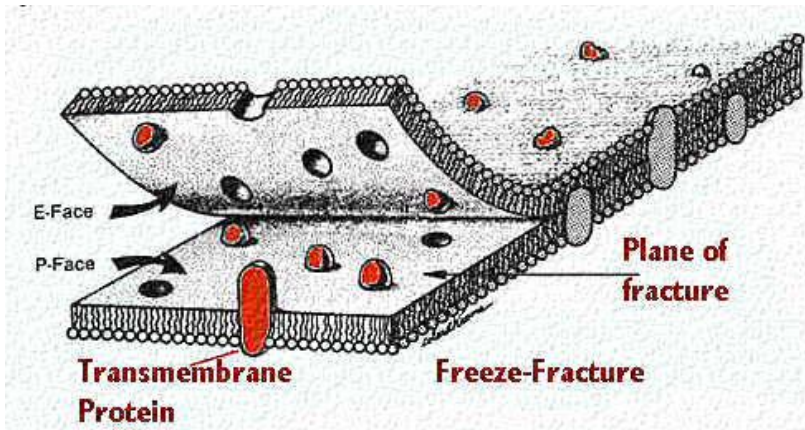
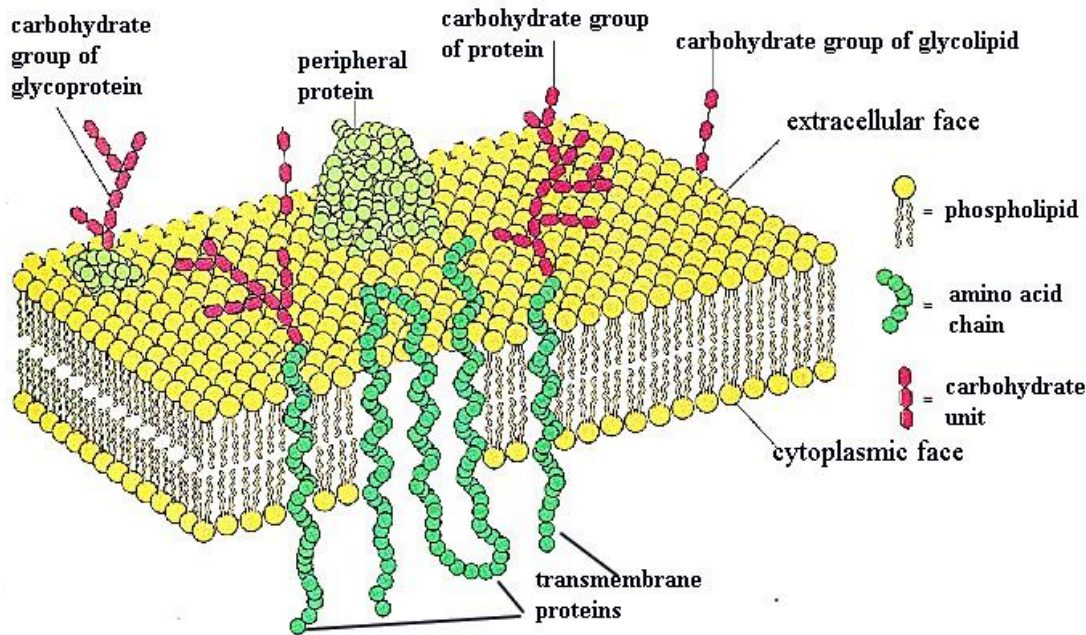


Figure 1-B-4. Structures of some sugar molecules.

N-acetylneuraminic acid (sialic acid)

The following figure shows trans membrane proteins passing through the lipid bilayer. Can you find the multipass and single-pass trans membrane proteins in this figure?



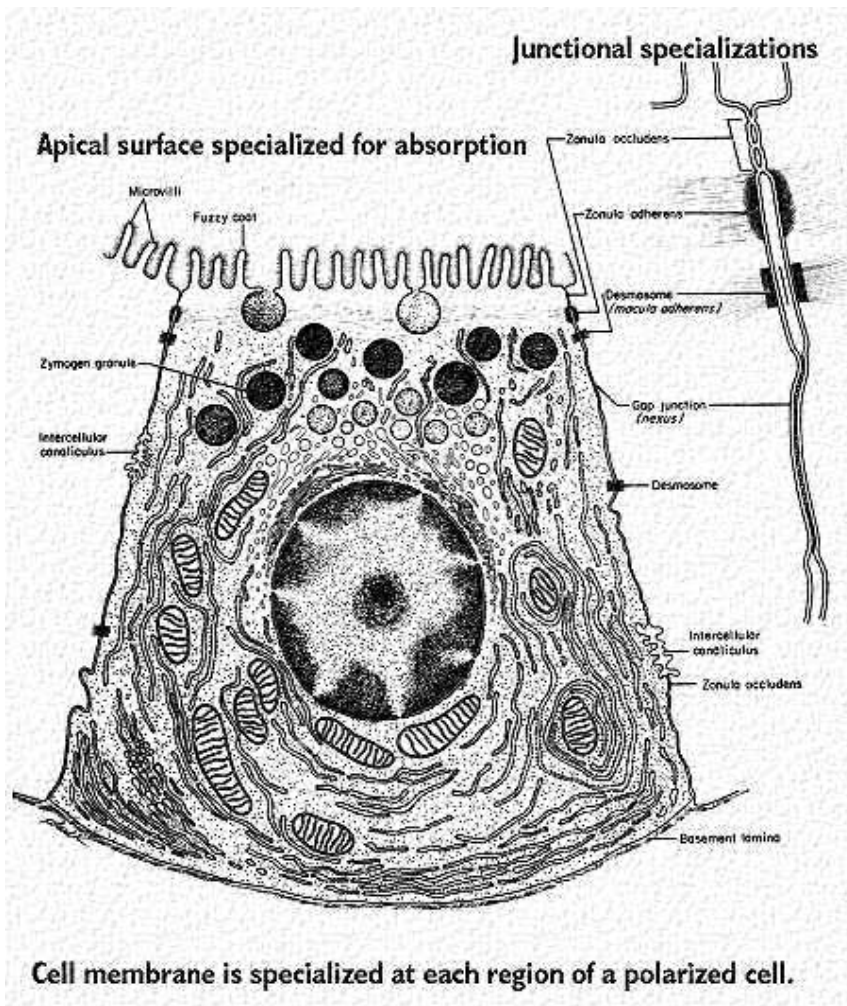
An EM view of membranes via freeze fracture/freeze etch .

You can best see protein distribution via a technique called freeze fracture/freeze etch. The freeze-fracture/freeze etch technique starts with rapid freezing of a cell. Then the frozen cells are cleaved along a fracture plane. This fracture plane is in between the leaflets of the lipid bilayer, as shown by this cartoon. The two fractured sections are then coated with heavy metal (etched) and a replica is made of their surfaces. This

replica is then viewed in an electron microscope. One sees homogeneous regions where there was only the exposed lipid leaflet (Is the exposed surface made of polar or nonpolar groups?)

In certain areas of the cell, one also sees protrusions or bumps. These are colored red in the cartoon. Sometimes one can see structure within the bumps themselves. These are the **transmembrane proteins**.

The following illustration will show you a freeze-fracture/freeze etch view. The organization or structure of the transmembrane proteins can often be visualized.



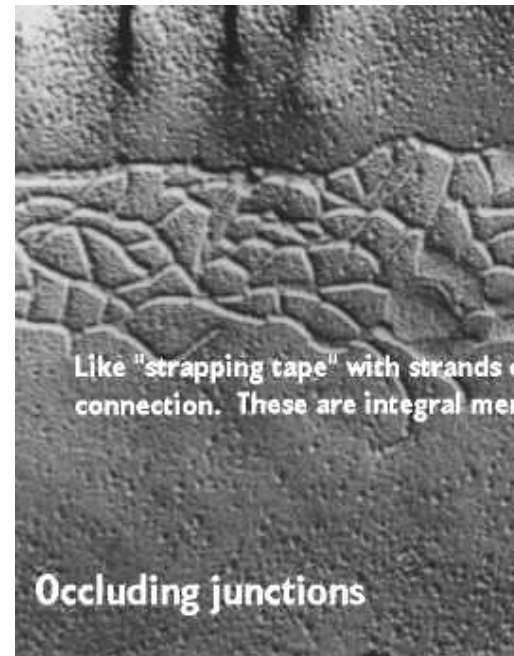
Cell membrane is specialized at each region of a polarized cell.

Membrane specializations (Adhesions): Junctions

The drawing is of a polarized cell. The top is specialized for absorption and the bottom for transfer of materials to the blood stream. The sides have specialized junctions that keep the nutrients from entering the space between the cells.

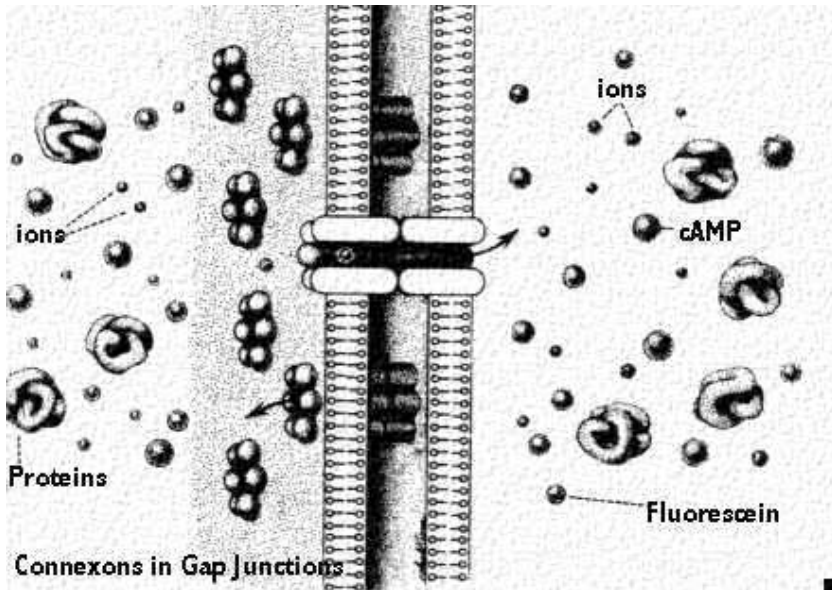
Tight Junctions

One of these is called a **tight junction** or "**occluding junction**" (**zonula occludens**). This is shown as the top junction in the above drawing. At this site, membrane glycoproteins and associated "glue" bind the cells together like double-sided "strapping tape". The freeze-fracture/freeze etch view of this junction (shown below) illustrates the ridges in the plane of the exposed leaflet. These are the proteins that bind to the proteins from the adjacent cell.



Gap Junctions

Another type of junction allows communication between cells. This type is called a **gap junction**. Small molecules or ions can pass through, as we will see by the following figures.



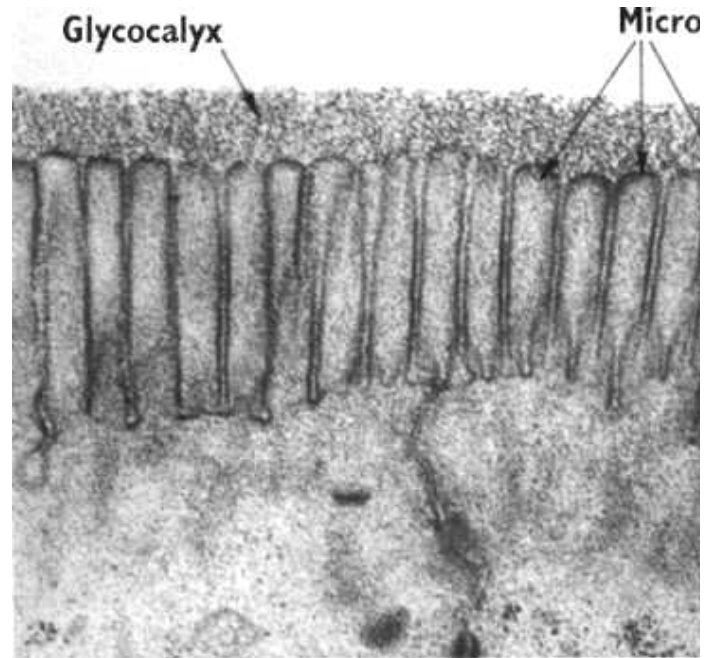
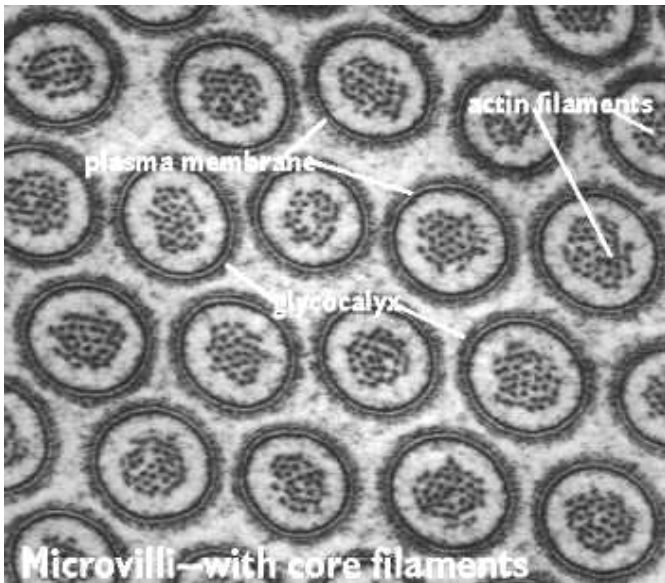
The above freeze-fracture / freeze etch image shows the internal view of the **gap junction** on the left. The proteins look like little donuts which reflects the fact that they are actually a channel. These proteins are "connexon" molecules. The side facing the cytoplasm (called the P face) is shown in the center panel. The region looks like aggregated lumps. Finally, the typical electron microscopic view is seen in the third panel. This shows a thin line between the two plasma membranes indicating a "gap junction".

There are several ways to prove the cells are communicating by gap junctions. First, one can identify the connexon molecules by immunocytochemical labeling. Second, one

can identify the actual junctional complex with freeze-fracture/freez etch. To see if they are functional, however, one needs to inject one cell with a dye and watch to see if it is transferred to another cell. This cartoon diagrams a view of a gap junction showing molecules that can freely pass. Ions pass and in this way the cells can be electrically coupled together. Other small molecules that pass through include cyclic AMP (a second messenger) and the dye marker fluorescein. This last compound enables the scientist to study transport through the gap junction.

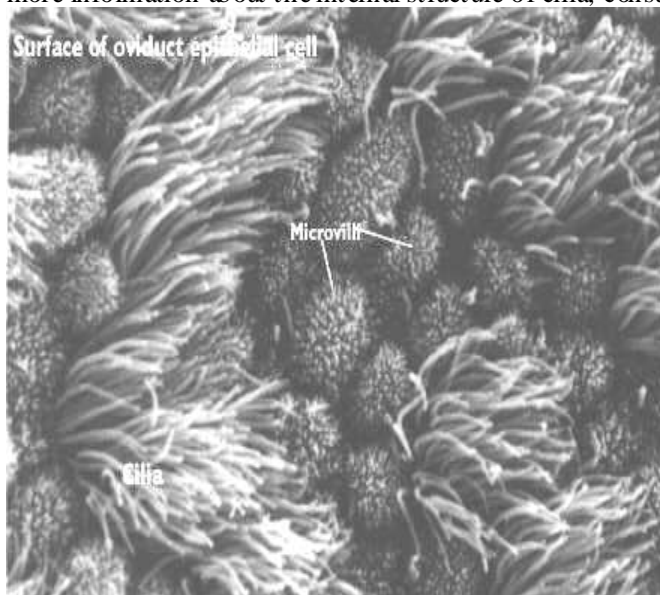
Membrane Specializations: Microvilli

The purpose of this final presentation is to introduce a surface specialization that projects from membranes called the microvillus. It is covered by a plasma membrane and encloses cytoplasm and microfilaments. Typically microvilli are found in absorptive cells, whenever there is a need for an increase in surface area.



It is also covered by a **glycocalyx which are peripheral glycoproteins** that attach themselves to the membrane. It might be used to trap nutrients, protect against toxic substances, or adhere to substances needed for uptake. Enzymes used for the cell's function are stored in this region, depending on the cell type. The figures to the right show views of microvilli cut transversely. Note that the microvilli are lined with the **Unit membrane**. (top figure) The core of filaments may allow them to move, although such movement is not as great as that of **cilia or flagella**

The lower figure shows a scanning electron micrograph of the luminal surface of the oviduct. It illustrates one difference between cilia and microvilli. The longer projections are cilia and the shorter projections are microvilli. For more information about the internal structure of cilia, consult the [Cilia Web page](#)



02 Red Cell Membrane Structure – Antigens

ANTIGEN - DEFINITION

An antigen is any substance, which in appropriate biological circumstances can stimulate an immune response; e.g. the formation of antibody or the activation of antigen specific effector cells.

WHAT ARE BLOOD GROUP ANTIGENS?

Blood group antigens are located within many red cell membrane chemical structures and are inherited characteristics. Inheritance of genetic material results in the production of different blood group system structures, i.e. ABO, Rh, etc. The presence of alternative genes (alleles) at these genetic loci result in the production of different antigen substances, e.g. the A or B antigens of the ABO blood group system. The different alleles at a particular gene locus have arisen due to genetic mutation, which occurs spontaneously. This provides the mechanism by which the diversity of red cell antigen structures are produced. The majority of these alternative structures do not however appear to affect red cell function. 'Red cell antigens' are therefore variable parts of red cell membrane extracellular protein or carbohydrate structures that are capable of eliciting an immune response.

The blood group genes, via mRNA, either code for red cell membrane proteins directly or code for enzymes that cause the production of specific red cell membrane carbohydrates (i.e. sugars).

BASIC RED CELL MEMBRANE COMPOSITION

The red cell membrane is composed of approximately equal amounts of lipid (approximately 44%) and protein (approximately 49%) together with a small amount (approximately 7%) of carbohydrate.

Lipid

The major lipid component (approximately 75%) of the red cell membrane is phospholipid, which are molecules having hydrophilic (water-soluble) polar ("head") groups and two hydrophobic (water insoluble) low viscosity chain ("tail") groups. The hydrophobic areas align to form a basic phospholipid bilayer structure, which is approximately 7nm across. This structure provides the red cell membrane with its major properties of impermeability (i.e. to ions, water and metabolites) and fluidity (i.e. flexibility and deformability within the plane of the membrane).

Protein

The presence of protein within the phospholipid bi-layer provides the capacity for selective transport across the membrane barrier, as well as providing a skeletal function. The protein may be extrinsic (projecting above the phospholipid bi-layer) or intrinsic (on the inside or across the phospholipid bilayer). Red cell protein may be free within the phospholipid bi-layer or anchored to the ankyrin and spectrin protein underneath the phospholipid bilayer.

Carbohydrate

The ABO, Lewis, P₁, I and H antigens are carbohydrate structures. The carbohydrate present in the red cell membrane is associated with either protein, (i.e. as glycoprotein), or with lipid (i.e. as glycolipid):

GLYCOPROTEIN

These are mainly long chain structures (e.g. sialoglycoprotein) extending above the red cell membrane surface. These molecules contain most of the red cell membrane sialic acid (i.e. N-acetyl-neuraminic acid). Sialic acid is one of the major charged molecules of the red cell membrane. Glycoprotein comprises the major charged molecules within the red cell membrane and as such it confers the red cell with a net negative charge.

The most common types of red cell sialoglycoprotein are glycophorin A (GPA), which includes the MN antigen structures and glycophorin B (GPB), which includes the Ss antigen structures. Since these rod-like glycoprotein structures extend some distance above the red cell membrane, they are (like the Duffy glycoprotein) sensitive to proteolytic enzyme treatment (e.g. by papain, etc.). The Rh, Kell, Kidd and Lutheran antigen structures are also glycoprotein.

GLYCOLIPID

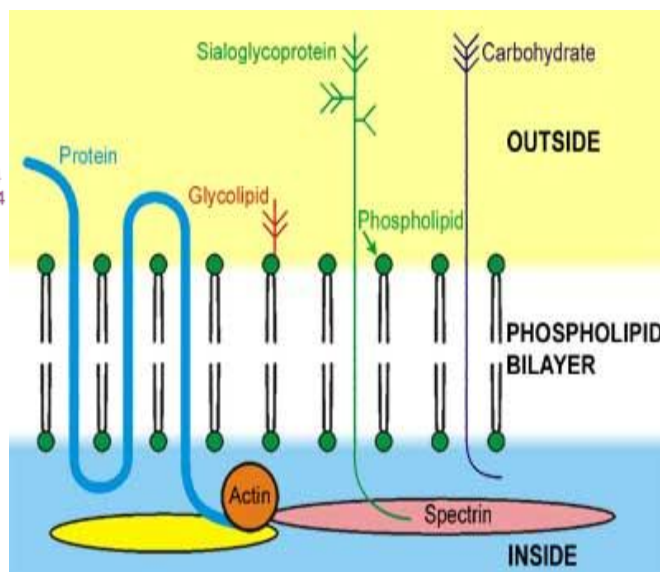
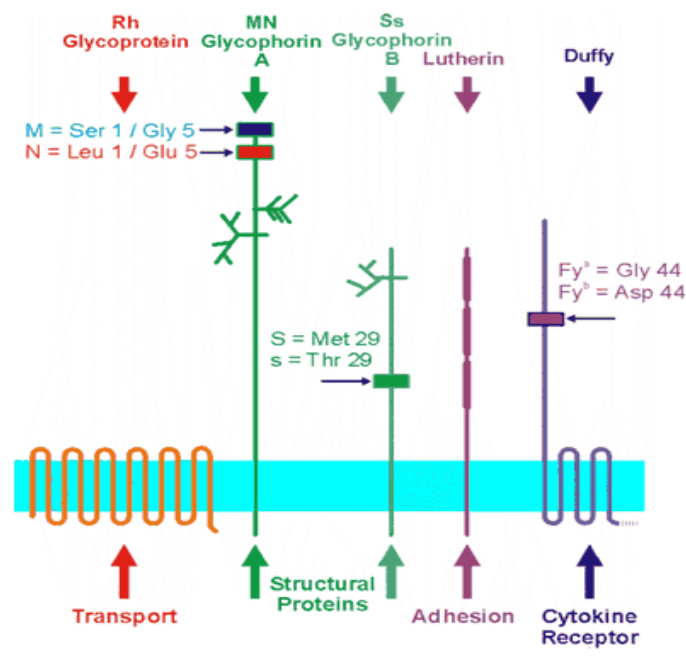
These molecules form only approximately 5% of the total lipid presence and are known as sphingosine molecules (i.e. long chain fatty acids).

INDIVIDUAL ANTIGENIC SPECIFICITIES

The difference between antithetical blood group antigens has been identified in some instances to be due to very minor differences within the 'parent' protein. The M and N antigen structure difference is produced by a change to two amino acids (at positions 1 and 5) within glycophorin A, a protein consisting of 131 amino acids. The difference between the S and s antigens is due to a single amino acid change at position 29 within glycophorin B, a protein consisting of 72 amino acids. These differences do not appear to affect the specific function of the different 'parent' glycoprotein structures (e.g. as a structural, receptor molecule, trans-membrane transport function, etc.).

The very minor structural variations associated with some antithetical antigens, though capable of eliciting an antibody response in some patients results (to some degree) in their relatively low immunogenicity. This situation is in sharp contrast to the immunogenicity of the RhD protein, which has no antithetical antigen option (i.e. D has no allelic gene option). As such, a D-recipient sees the large extracellular areas of the RhD protein (consisting of 417 amino acids) as foreign. This accounts for the high immunogenicity of the RhD protein, compared with other (non-ABO) red cell antigens.

Diagrammatic representation of some of the major red cell membrane glycoproteins and variations in antigenic structures (not to scale)



Diagrammatic cross-sectional representation of the red cell membrane structures (not to scale)

Blood group antigens are associated with a variety of red cell membrane structures, i.e.

NAME	ASSOCIATED MEMBRANE STRUCTURE *	CHROMOSOME GENE LOCUS
ABO	Carbohydrate	9
MNS	Sialoglycoprotein (GPA/ GPB)	4
P	Glycolipid	22
Rh	Proteins	1
Lutheran	Glycoprotein	19
Kell	Glycoprotein	7
Lewis	Carbohydrate	19
Duffy	Glycoprotein	1
Kidd	Glycoprotein	18

* For further information regarding the functions associated with these red cell membrane structures, refer to the individual blood group systems.

Therefore 'red cell antigens' can be identified to be associated with specific red cell membrane structures, the majority of which have been identified to have a specific function. Since antigen specificity may be defined by something as small as a single carbohydrate (e.g. A and B antigens) or alternative amino-acids within a single protein (e.g. M and N antigens), each red cell can have an enormous number of each antigen expressed within its membrane, i.e.

BLOOD GROUP	ANTIGEN	APPROXIMATE NUMBER OF ANTIGEN SITES PER RED CELL *
ABO	A	810,000 - 1,200,000
	B	600,000 - 850,000
Rh	D	10,000 - 40,000
Kell	K	3,000 - 4,000
Duffy	Fy ^a	10,000 - 15,000

* As calculated by radioisotope (125I) labelled antibody binding and flow cytometry experiments (performed by different research groups).

Generally, the same antibody binding experiments have demonstrated that there are more antigen sites detectable on red cells of the homozygote rather than the heterozygote genotype, i.e.

DD homozygote: approximately 25,000 - 37,000 RhD antigen sites / red cell.

Dd heterozygote: approximately 10,000 - 15,000 RhD antigen sites / red cell.

This factor is a major reason why homozygous antigen expression is preferred for the red cells that are used for antibody detection, since they offer a potential for improved reactivity (i.e. more antigen available for the antibody to react with). The location and number of antigen sites on each red cell can affect antigen-antibody reactivity in a variety of ways:

1. The type of laboratory technique used to demonstrate the antigen-antibody reaction (i.e. IgG ABO antibodies are able to work in a saline medium, whereas AHG and/or enzyme techniques must be used to detect IgG Rh antibodies).
2. The effects of the action of enzymes (e.g. papain) on the antigen-antibody reaction, i.e. as to whether antigen reactivity is enhanced (e.g. Rh) due to the removal of adjacent (interfering) proteins or destroyed (e.g. Duffy) due to the antigen structure being removed by the enzyme treatment.
3. The strength (avidity) of the antigen-antibody reaction produced, i.e. the ease with which the antibody is able to react with its antigen.

DEFINITION

Antibodies are protein molecules (immunoglobulins) occurring in body fluids, which are produced in response to the introduction of a foreign antigen. The antigen-antibody reaction is specific, i.e. a given antibody molecule has the property of specific combination with the structure (antigen) which elicited its formation.

ANTIBODY PRODUCTION

Antibody molecules are produced when an antigenic structure is recognised by the immune system as foreign. This process involves a series of interrelated stages, which forms the 'humoral' rather than 'cellular' part of the immune response mechanism. Basically, an antigen presenting cell (APC), normally a macrophage processes the antigenic material. The processed antigen (or antigen fragment) is presented by the APC, together with a glycoprotein coded for by the Major Histocompatibility Complex (MHC), to CD4+ (helper) T lymphocyte. These in turn inter-react, resulting in the activation of an existing B lymphocyte into growth and differentiation to become a plasma cell. It is the plasma cells that synthesise and secrete antibody molecules that are specific for the antigen structure that stimulated their production. The amount and type / subtype of immunoglobulin produced results from the interaction of CD4+ (T-helper) and CD8+ (T-suppressor) lymphocytes. The antibody synthesis is specific for the structure of the stimulating antigen. This results in the production of a 'unique' antibody specificity, i.e. during B cell differentiation there is a rearrangement of genes determining heavy and light chain structure, producing a unique immunoglobulin for each B cell (i.e. the "lock and key" theory).

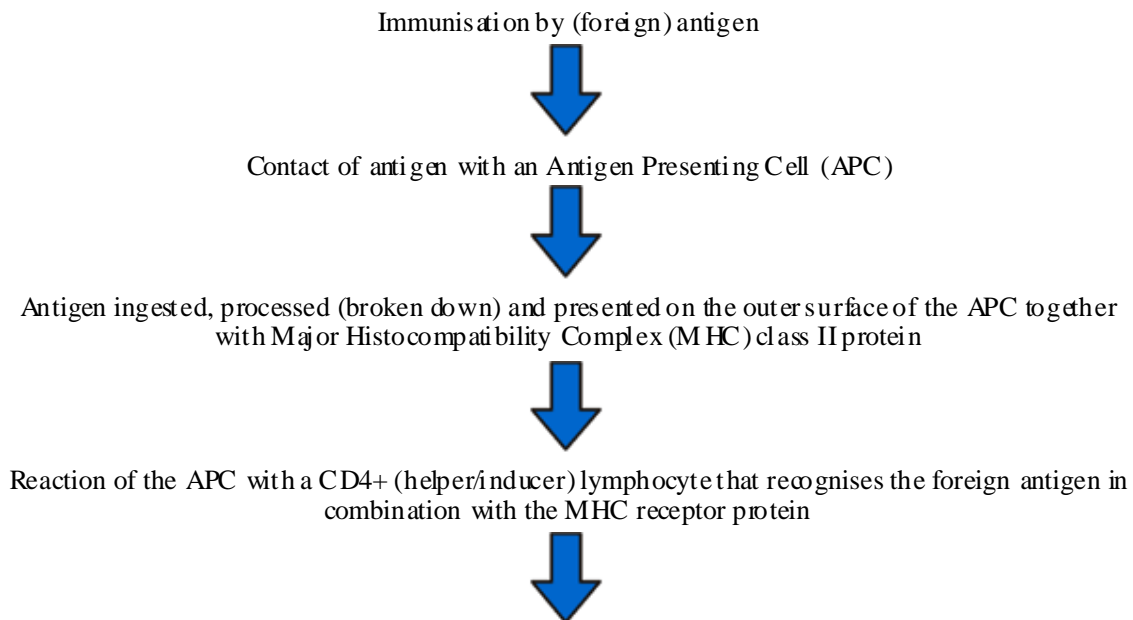
A (large) number of B lymphocytes may be involved in each immune response, since red cell antigens are complex structures containing different portions. In the transfusion laboratory since these (anti-epitope polydonal) antibodies are identified for practical purposes as 'an antibody' against a single red cell antigen specificity.

The production of antibody, involving circulating monocytes, T and B lymphocytes and tissue bound macrophages, may result in either a primary and/or secondary response.

Primary Response

This type of immune response is produced as a result of the first antigen encounter, and 'classically' results in the production, after a relatively lengthy delay (e.g. several days to months), of small amounts of IgM antibody, with the production subsequently changing over to IgG antibody molecules. However, this is a generalisation with regard to the primary immune response to some red cell antigens (e.g. RhD) may involve the relatively rapid production of mainly IgG antibody. The primary immune response requires the involvement of T lymphocytes, APC and B lymphocytes. Once antibody has been produced, some B cells have the capability to act as long lived primed ("memory") cells, which remain in the circulation.

Summary of primary immune response process Immunisation by (foreign) antigen



APC secretes interleukin-1 (IL-1) stimulating the CD4+ cell to secrete lymphokines and interferon which stimulate proliferation of more T lymphocytes - though this may be inhibited by activation of CD8+ (suppressor) T lymphocytes



Secretion of B cell growth factor (BCGF) and binding to the activated CD4+ cell stimulates a B lymphocyte to divide and produce genetically identical daughter cells



Daughter cells develop into plasma (antibody secreting) cells and memory cells

Secondary ('anamnestic') Response

This type of response is produced by the 'primed' B-lymphocytes (memory cells) due to the exposure of a second dose of the same antigen, i.e. at a later time from that which resulted in the primary immune response. There appears to be a need for a time delay between the two antigenic exposures for the secondary response to be produced. The secondary response is independent of T cell involvement.

In general (though not always) the secondary response results in the production of larger amounts of IgG (rather than IgM) antibody, with little time delay. In addition, certain B cell clones undergo somatic mutation and start to produce an antibody with improved affinity for the antigen, i.e. 'tailors' the antibody to produce a 'better fit' with its antigen, thereby increasing its reactivity (avidity). This process is called 'B-cell maturation'. Generally, the decline in antibody production is slower than that seen in the primary response and may frequently continue at a high level for many years.

The immune response in man that results in the production of antibody to a red cell antigen is highly individualistic, being dependent upon a variety of factors. These include the amount and immunising capability ('immunogenicity') of the antigen, the immune responsiveness of the patient and a number of genetic effects, i.e. the type of HLA-DR (Class II) alleles present. These factors therefore determine if a patient actually produces an antibody as a result of a transfusion (or pregnancy), resulting from the introduction of antigen positive red cells into the patient's antigen negative circulation. This may, for example, result in some people producing no antibody even after repeated antigen stimulation. These people have in the past been categorised as "non-responders". Conversely, some people quickly produce multiple blood group antibody specificities following a single stimulation; i.e. these people have been classified as "responders".

All alloantibodies are produced in response to an overt antigenic stimulus. However, antibodies to red cell antigens may be stimulated by either of the following two circumstances:

a. Antibody production in response to environmental antigen

Chemical structures identical or very similar to some (especially carbohydrate) blood group antigens are very common in nature (i.e. present on bacteria and/or viral membranes and in food). These are therefore introduced into the body independently of an immunisation by red cells. Antibodies are produced to these structures which are then described as antibodies to "red cell antigens" (i.e. because although they are produced by non-red cell antigen structures, they react with the same structures within red cell membranes and are detectable in tests using red cells as targets).

This mechanism accounts mainly for the production of antibodies to the A and B antigens of the ABO system (i.e. anti-A, anti-B and anti-A,B), but may also result in the production of other apparent red cell antigen specificities (e.g. anti-K, anti-E, etc.). As a result, since these antibodies are produced in the absence of actual immunisation by foreign antigen positive red cells. Transfusion scientists have referred to their production as being either "Naturally Occurring" or "Naturally Acquired".

b. Antibody production in response to red cell antigen

The red cells from one person may typically be introduced into the circulation of another person by two mechanisms, namely transfusion or pregnancy. Either process can result in antibody production due to the foreign red cell antigen stimulating the

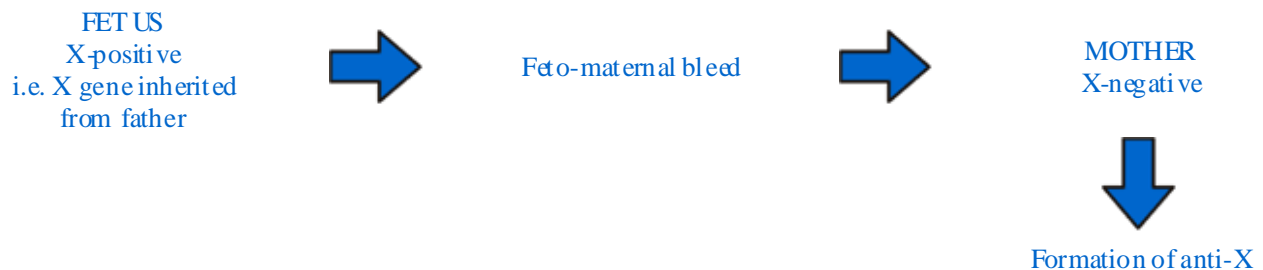
person's immune response. These are called 'immune' red cell antibodies, i.e. alloantibodies. The production of an immune antibody against a red cell antigen as a result of transfusion is a relatively rare event, i.e. only approximately 2-9% of patients.

The basic mechanisms of immune antibody production to a red cell antigen 'X' may be demonstrated diagrammatically as follows:

TRANSFUSION



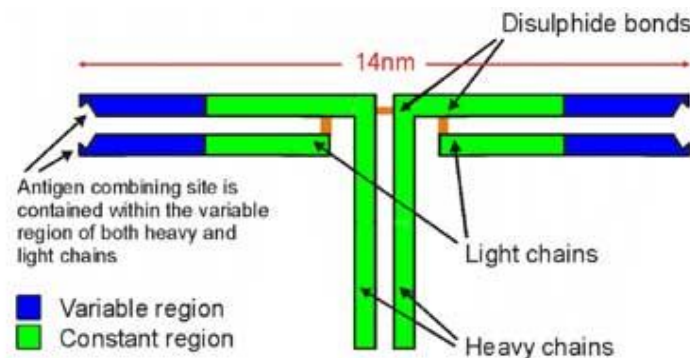
PREGNANCY



ANTIBODY STRUCTURE

Each immunoglobulin molecule is basically composed of four polypeptide chains, having two pairs of identical heavy and light protein chain types held together by non-covalent interactions and disulphide bonds.

Basic (simplified) structure of unbound IgG immunoglobulin



There are five possible variations of heavy chain types, i.e. gamma (G or Γ), mu (M or μ), alpha (A or Λ), delta (D or Δ) and epsilon (E or Σ), which determine the five 'classes' of immunoglobulins, i.e. IgG, IgM, IgA, IgD and IgE respectively. These chain types differ in length, carbohydrate content (glycosylation), etc., resulting gamma chain type has four variations, producing four sub-types of IgG (i.e. IgG1, IgG2, IgG3 and IgG4) that also results in variations in their biological activity.

Most IgG blood group antibodies are of sub-types IgG1 and IgG3, and only rarely are they IgG2 or IgG4. There are two classes of IgA, i.e. IgA1 and IgA2.

There are two types of light chain, kappa (K or κ) and lambda (L or λ). The light chains of antibody molecules produced by each single clone of immunocytes will be the same types. Blood group antibodies present in serum/plasma are invariably either of the type IgG or IgM, being only rarely IgA and never IgD or IgE.

Basic characteristics of the immunoglobulin classes IgG and IgM

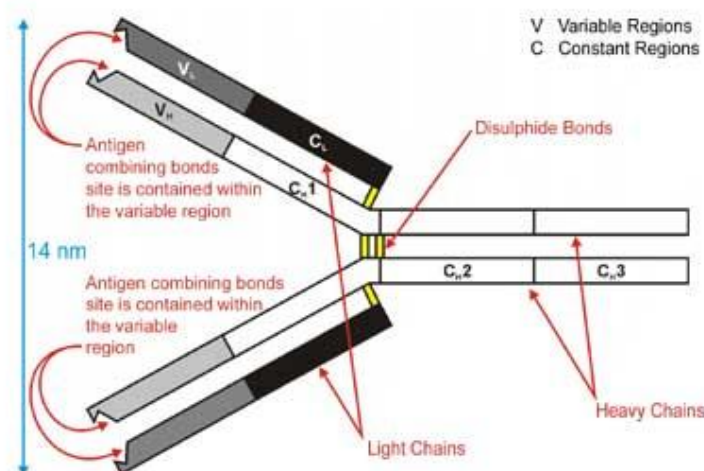
CHARACTERISTIC	IgG	IgM
Type of heavy chain	γ	μ
Type of light chain	κ or λ	κ or λ
Approximate molecular weight	160,000	900,000
Number of antigen combining sites per molecule	2	10
Normal serum concentration (mg/dl)	800 - 1,700	50 - 190
Percentage of total immunoglobulin	70 - 80%	5 - 10%

Basic properties of the immunoglobulin classes IgG and IgM

PROPERTY	IgG	IgM
Placental transfer	Yes	No
Complement activation	Yes	Yes
Treatment with dithiothreitol (DTT)	Unaffected	Reduced
Normal reaction temperature	37°C	4°C - 20°C
Primary immune response involvement	Rare	Yes
Secondary immune response involvement	Yes	Rare

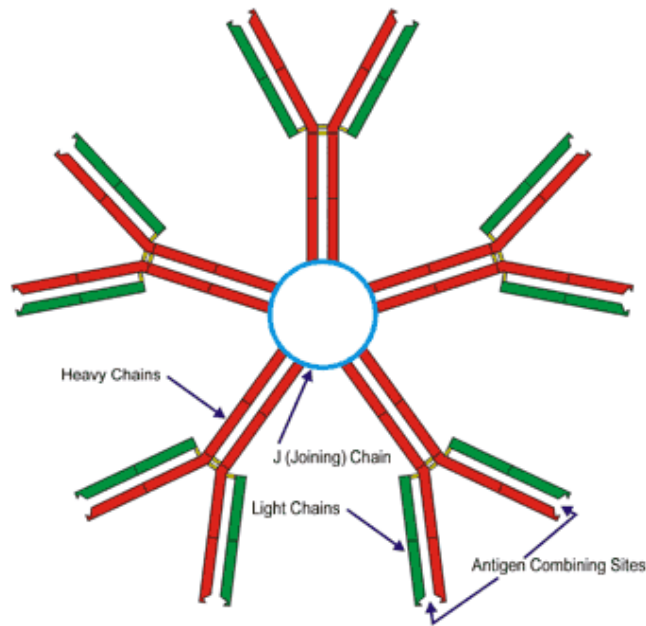
SIMPLIFIED IMMUNOGLOBULIN STRUCTURES

The two light chains and two γ heavy chains of an IgG molecule are held together by disulphide bonds between cysteine amino acids (and by noncovalent hydrophobic interactions).



The IgM molecule is a pentameric form with the five sections, each comprising two light chains and two μ heavy chains, being held together by a J-chain.

Basic (simplified) structure of the IgM immunoglobulin

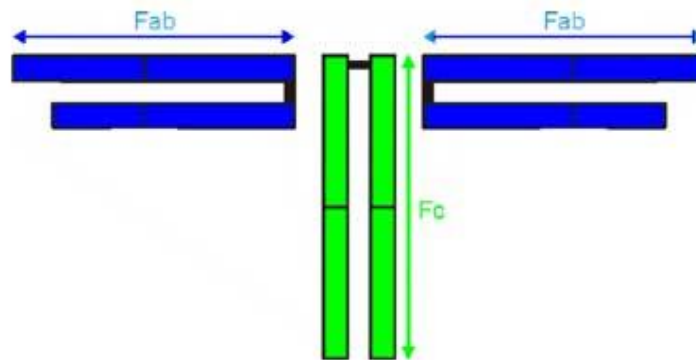


Functional Areas in IgG immunoglobulin molecule may be broken down to identify its functional areas by the use of sufficiently strong concentrations of proteolytic enzymes. At these concentrations, the action of the enzyme papain produces two Fab fragments and one Fc fragment, whereas pepsin acts to produce one F(ab)₂ fragment (essentially two joined Fab fragments) and two free carboxy-terminal chains.

The Fab (Fragment Antigen Binding) fragment is composed of an intact light chain and the amino-terminal end of the heavy chain, linked together by interchain disulphide bonding. The Fab portion has been shown to contain specific antigen binding ability, i.e. each Fab portion contains one antigen binding site.

The Fc (Fragment Crystalline) fragment is composed of a dimer of the carboxy terminal portions of the two heavy chains linked by disulphide bonding and is associated with some of the IgG molecule's biological functions (e.g. complement activation and macrophage binding). The Fc fragment of the IgG molecule contains most of the carbohydrate content.

Enzyme (papain) digestion of an IgG molecule



Therefore, both heavy and light chains can be divided into specific areas, which are known to have specific functions, as follows:

Variable section

This section, at the amino-terminal end of the polypeptide chains (which is approximately 110 amino acids in length), determines the specificity of the antibody. It is composed of variable amino acid sequences, containing "hypervariable"

regions, which are concerned with antigen binding. This variability enables the immune response to generate the vast number of unique antibody specificities capable of reacting with the enormous diversity of potential antigens to which it may be exposed. For example, there are between 500- 1000 heavy chain and over 200 light chain variable region genes, with the capability of producing 10 million potential specificities.

Constant section

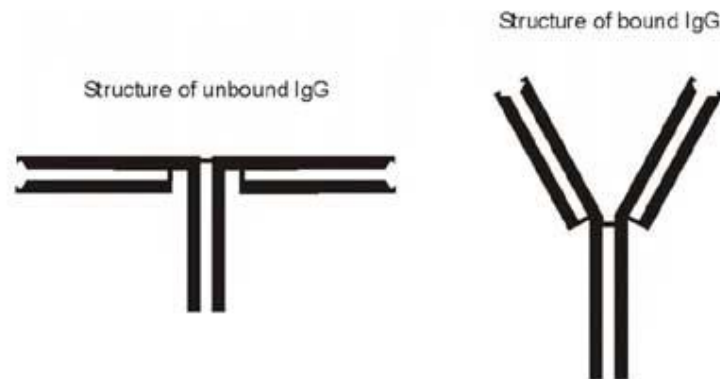
As the name suggests, this section of amino acids at the carboxy-terminal end of the polypeptide chain is virtually identical for a given class or sub-class of immunoglobulin. This section determines some of the biological functions of the antibody, e.g. complement activation, placental transfer and the ability to bind to effector cells (macrophages).

This region also contains the immunoglobulin 'serum groups' (e.g. the Gm groups, which are genetically determined differences in the amino-acid sequence of the \square heavy chains of different IgG subtypes and between the same IgG subtype of different people).

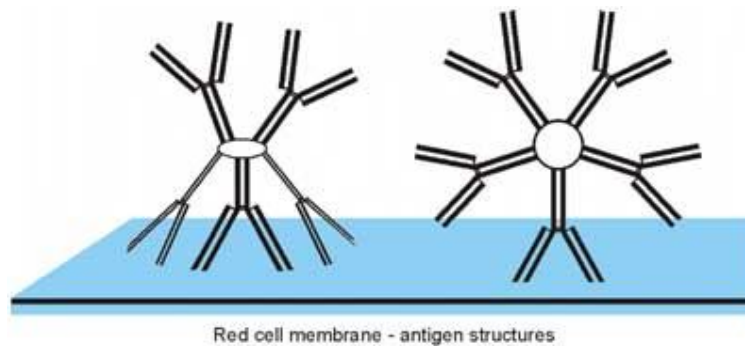
Hinge region

This is located within the constant section of the heavy chains and centres around two closely associated triplets of proline amino acids. This region provides the gamma heavy chain a degree of flexibility, enabling it to change its shape. An IgG molecule maintains a 'T' shape in serum/plasma enabling the antigen binding sites to be maximally distant from each other at 14nm. The IgG immunoglobulin becomes a 'Y' shape on binding with its antigen. This allows greater accessibility of the constant region (e.g. for complement activation).

Most IgM molecules appear symmetrical, with a diameter of approximately 30nm. These molecules, as well as being flexible at the hinge regions of each of the individual pentameric components, changing shape on binding with an antigen (like IgG molecules), also have the capability of assuming various "crab-like" shapes due to movement at the J-chain binding sites.



Examples of possible variable structures of bound IgM molecules

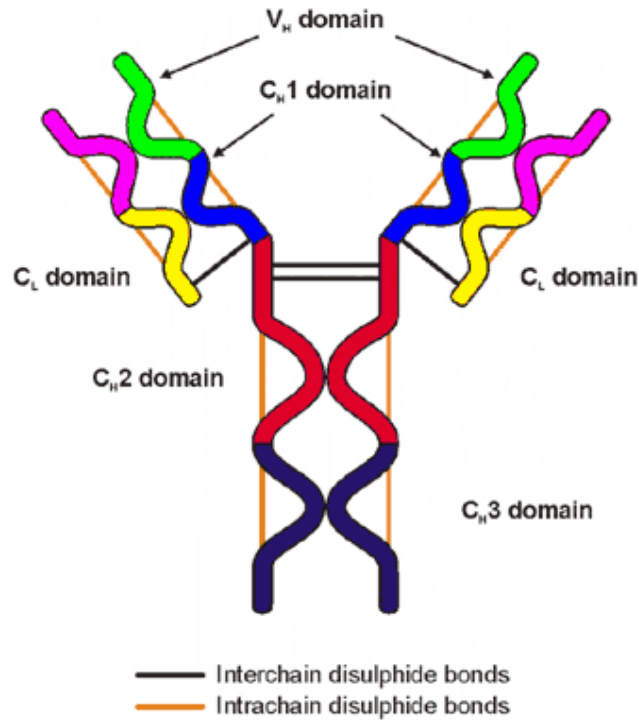


Domain regions

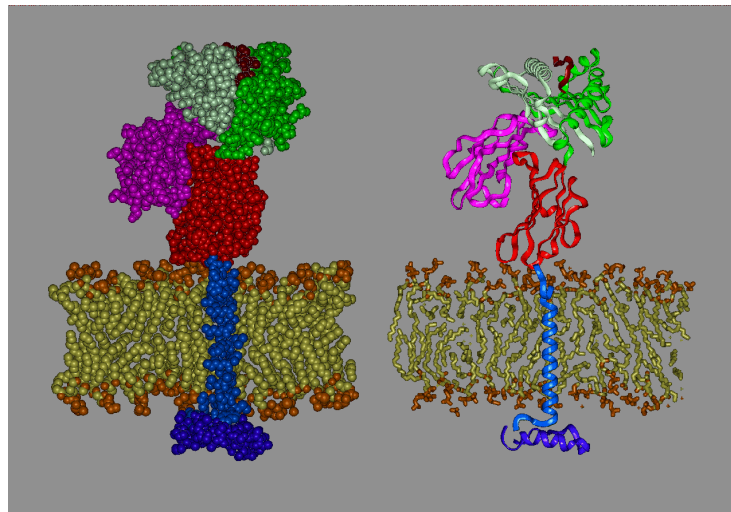
In their native states, the polypeptide chains of immunoglobulin molecules do not exist as linear sequences of amino acids but are folded, by intrachain disulphide bonds, into globular regions or domains. Extensive folding of the variable regions of both

heavy and light chains brings the hyper-variable amino acid regions (which determine the antibody specificity) into close proximity to the antigen structure. This enables the immunoglobulin to form a structure to enable maximum contact with antigen.

Simplified domain structure of an IgG molecule



Structure and Function of the Major Histocompatibility Complex (MHC) Proteins.



The Immune System

The continuing health of an animal depends upon its ability to recognise and repel disease; this ability is called **immunity**.

Two types of immunity exist, innate and adaptive.

Innate immunity, a first line of defence, is furnished by barriers such as skin, tears, saliva, and mucus, and the tissue inflammation that occurs after injury or infection.

Adaptive immunity develops specific defences against an invader that can be invoked whenever this particular intruder attacks again.

Forms of adaptive immunity.

The immune system responds to surface structures of the invading organism called antigens. There are two types of adaptive immune responses: **humoral** and **cell mediated**.

In **humoral** immune responses antibodies appear in the body fluids and stick to and destroy antigens. The response is to toxic substances outside of the cell.

In the **cell-mediated** immune response cells that can destroy other cells become active (**T-cells**). They destroy disease infected cells or cells making mutant forms of normal molecules.

Cell-mediated response molecules.

When disease associated proteins occur in a cell they are broken into pieces by the cell's proteolytic machinery. Cell proteins become attached to antigen fragments and transport them to the surface of the cell, where they are "presented" to the body's defence mechanisms.

These transport molecules are called the Major Histocompatibility Complex (**MHC**) proteins. Without these, there would be no presentation of internal or external antigens to the **T cells**. The importance of MHC proteins is that they allow T cells to distinguish self from non-self. In every cell in your body, antigens are constantly broken up and presented to passing T cells. Without this presentation, other aspects of the immune response cannot occur.

Class I MHC proteins (found on all nucleated cell surfaces) present antigens to cytotoxic T lymphocytes (**CTLs**). Most CTLs possess both T-cell receptors (**TCR**) and CD8 molecules on their surfaces. These TCRs are able to recognize peptides when they are expressed in complexes with MHC Class I molecules. For the TCR to bind a peptide-MHC complex two conditions must be met. Firstly, the TCR must have a structure which allows it to bind the peptide-MHC complex. Secondly, the accessory molecule CD8, must bind to the alpha-3 domain of the MHC Class I molecule. Due to genetic recombination events each CTL expresses a unique TCR which only binds a specific MHC-peptide complex. CTLs which recognize self-peptides (i.e. peptides produced by the normal host body as opposed to a foreign or cancerous cells) are removed in the thymus or tolerized after their release from the thymus. So, if a CTL can bind to a MHC-peptide complex on the cell surface, that cell is producing a peptide which is not native to the host.

The MHC Class II proteins (found only on B lymphocytes, macrophages, and other cells that present antigens to T cells), which primarily present peptides, which have been digested from external sources, are needed for T-cell communication with B-cells and macrophages. Class II MHC proteins presenting antigens are detected by a different group of T cells (called T-helper or TH cells) to Class I MHC proteins (which are detected by CTLs cells).

The MHC proteins, and several closely associated with them in the carrying out of their functions, are coded for by loci that are close together within the **Human Genome**. Major Histocompatibility Complex proteins and their associated molecules are fundamental in the process of antigen presentation.

DEFINITIONS:

- **Histocompatibility (transplantation) antigens:** Antigens on tissues and cells that determine their rejection when grafted between two genetically different individuals
- **Major histocompatibility (MHC) antigens:** Histocompatibility antigens that cause a very strong immune response and are most important in rejection
- **MHC complex:** Group of genes on a single chromosome encoding the MHC antigens
- **HLA (human leukocyte antigens):** MHC antigens of man (first detected on leukocytes)
- **H-2 antigens:** MHC antigens of mouse

Types of graft

- **Xenograft:** Grafts between members of different species (also known as heterologous, xenogenic or heterografts)
- **Allograft:** Grafts between two members of the same species (also known as allogenic or homograft)

- **Isograft:** Grafts between members of the same species with identical genetic makeup (identical twins or inbred animals)

Haplotype: a group of genes on a single chromosome

PRINCIPLES OF TRANSPLANTATION

An immunocompetent host recognizes the foreign antigens on grafted tissues (or cells) and mounts an immune response which results in rejection. On the other hand, if an immunocompromised host is grafted with foreign immunocompetent lymphoid cells, the immunoreactive T-cells in the graft recognize the foreign antigens on the host tissue, leading to damage of the host tissue.

Host-versus-graft-reaction

The duration of graft survival follows the order, xeno- < allo- < iso- = auto-graft. The time of rejection also depends on the antigenic disparity between the donors and recipient. MHC antigens are the major contributors in rejection, but the **minor histocompatibility antigens** also play a role. Rejection due to disparity in several minor histocompatibility antigens may be as quick or quicker than rejection mediated by an MHC antigen. As in other immune responses, there is immunological memory and secondary response in graft rejection. Thus, once a graft is rejected by a recipient, a second graft from the same donor, or a donor with the same histocompatibility antigens, will be rejected in a much shorter time.

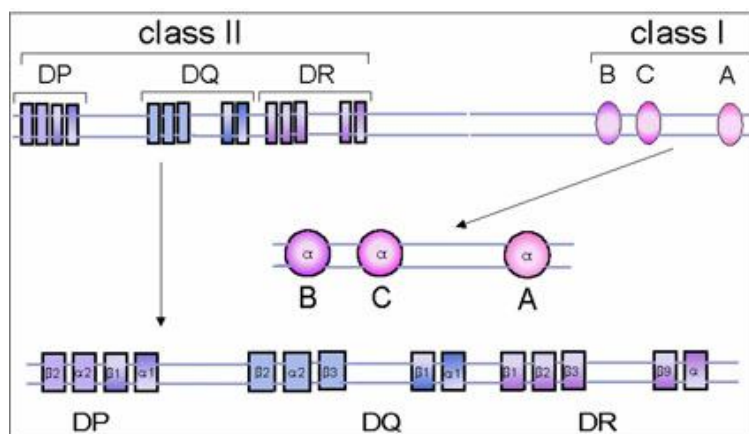
Graft-versus-host (GVH) REACTION

Histocompatible lymphoid cells, when injected into an immunocompromised host, are readily accepted. However, the immunocompetent T lymphocytes among the grafted cells recognize the all antigens and, in response, they proliferate and progressively cause damage to the host tissues and cells. This condition is known as graft-versus-host (GVH) disease and is often fatal. Common manifestations of GVH reaction are diarrhea, erythema, weight loss, malaise, fever, joint pains, etc. and ultimately death.

THE MHC GENE COMPLEX

The MHC complex contains a number of genes that control several antigens, most of which influence allograft rejection. These antigens (and their genes) can be divided into three major classes: **class I**, **class II** and **class III**. The class I and class II antigens are expressed on cells and tissues whereas class III antigens are represented on proteins in serum and other body fluids (e.g. C4, C2, factor B, TNF). Antigens of class III gene products have no role in graft rejection.

Human MHC



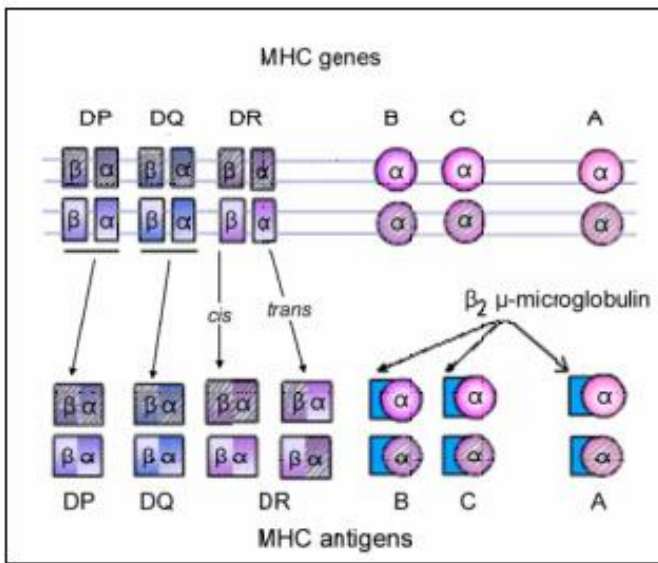


Figure 6b

The human MHC is located on chromosome 6.

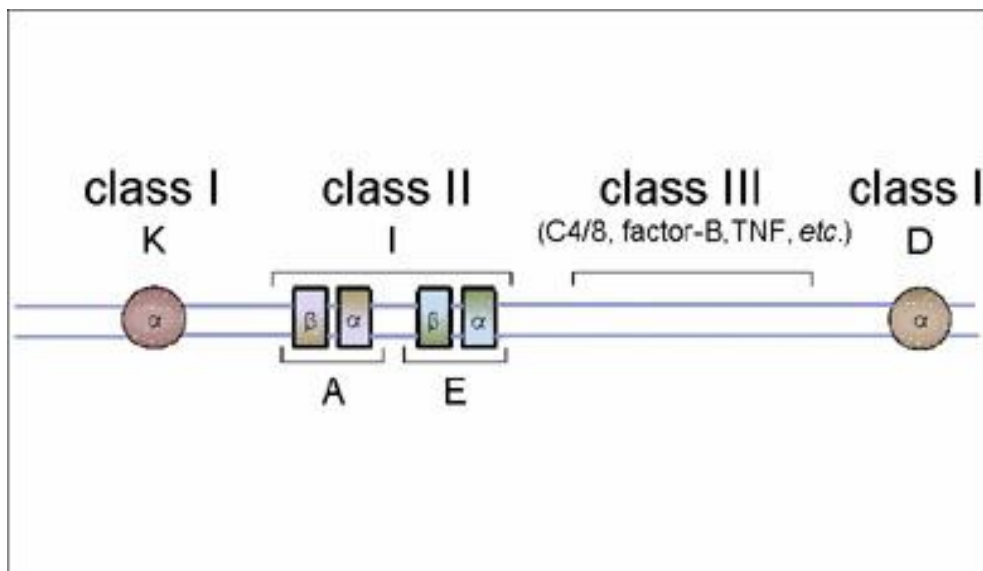
Class I MHC

The class I gene complex contains three major loci, **B**, **C** and **A** and other undefined minor loci (figure above). Each **major** locus codes for a polypeptide; the alpha-chain that contains antigenic determinants, is **polymorphic** (has many alleles). It associates with beta-2 microglobulin (beta-chain), encoded by a gene outside the MHC complex, and expressed on the cell surface. Without the beta-2 microglobulin, the class I antigen will not be expressed on the cell surface. Individuals with a defective beta-2 microglobulin gene do not express any class I antigen and hence have a deficiency of cytotoxic T cells.

Class II MHC

The class II gene complex also contains at least three loci, **DP**, **DQ** and **DR**; each of these loci codes for one alpha- and one beta-chain polypeptide which associate together to form the class II antigens. Like the class I antigens, the class II antigens are also polymorphic. The DR locus may contain more than one, possibly four, functional beta-chain genes.

Mouse MHC



The mouse MHC is located on chromosome 17.

Class I MHC

This consists of two major loci, **K** and **D**. Unlike the human MHC, the mouse class I gene complexes loci are not together but they are separated by class II and class III genes (Figure 6A).

Class II MHC

The class II gene complex contains two loci, **A** and **E**, each of which code for one alpha and one beta chain polypeptide, which form one class II molecule. The mouse class II gene complex is also known as the **I region** and the genes in this complex are referred to as **Ir** (immune response) genes since they determine the magnitude of immune responsiveness of different mouse strains to certain antigens. Products of the A and E loci are also termed IA and IE antigens, collectively known as Ia antigens.

MHC ANTIGENS

Nomenclature

HLA specificities are identified by a letter for locus and a number (A1, B5, *etc.*) and the haplotypes are identified by individual specificities (e.g., A1, B7, Cw4, DP5, DQ10, DR8). Specificities which are defined by genomic analysis (PCR), are names with a letter for the locus and a four digit number (e.g. A0101, B0701, C0401 etc).

Specificities of mouse MHC (H-2) are identified by a number. Since laboratory mice are inbred, each strain is homozygous and has a unique haplotype. The MHC haplotype in these strains is designated by a 'small' letter (a, b, d, k, q, s, *etc.*); for example, the MHC haplotype of B_{al}b/c mice is H^{2d}.

Inheritance

MHC genes are inherited as a group (**haplotype**), one from each parent. Thus, a heterozygous human inherits one paternal and one maternal haplotype, each containing three class-I (B, C and A) and three class II (DP, DQ and DR) loci. A heterozygous individual will inherit a maximum of 6 class I specificities (Figure 6: top). Similarly, the individual will also inherit DP and DQ genes and express both parental antigens. Since the class II MHC molecule consists of two chains (alpha and beta), with some antigenic determinants (specificities) on each chain, and DR alpha- and beta-chains can associate in either *cis* (both from the same parent) or *trans* (one from each parent) combinations, an individual can have additional DR specificities (Figure 6B). Also, there are more than one functional DR beta-chain genes (not shown in the figure). Hence, many DR specificities can be found in any one individual.

Crossover

Haplotypes, normally, are inherited intact and hence antigens encoded by different loci are inherited together (e.g., A2; B27; Cw2; DPw6; DQw9; DRw2). However, on occasions, there is crossing over between two parental chromosomes, thereby resulting in new **recombinant** haplotypes. Thus, any one specificity encoded by one locus may combine with specificities from other loci. This results in vast heterogeneity in the MHC make-up in a given population.

MHC antigen expression on cells

MHC antigens are expressed on the cell surface in a **co-dominant** manner: products of both parental genes are found on the same cells. However, not all cells express both class I and class II antigens. While class I antigens are expressed on all nucleated cells and platelets (and red blood cells in the mouse), the expression of class II antigens is more selective. They are expressed on B lymphocytes, a proportion of macrophages and monocytes, skin associated (Langerhans) cells, dendritic cells and occasionally on other cells.

MHC detection by serological test

The MHC class I antigens are detected by serological assays (Ab and C). Tissue typing sera for the HLA were obtained, in the past, from **multiparous** women who were exposed to the child's paternal antigens during parturition and subsequently developed antibodies to these antigens. More recently, they are produced by monoclonal antibody technology.

MHC detection by mixed leucocyte reaction (MLR)

It has been observed that lymphocytes from one donor, when cultured with lymphocytes from an unrelated donor are stimulated to proliferate. It has been established that this proliferation is due to a disparity in the class II MHC (DR) antigens and T cells of one individual interact with allogeneic class II-MHC antigen-bearing cells. (B cells, dendritic cells, Langerhans cells etc.) This reactivity was termed **mixed leukocyte reaction (MLR)** and has been used for typing some class II MHC antigens.

The test lymphocytes were mixed with irradiated or mitomycin-C treated homozygous leukocytes, containing B-lymphocytes and monocytes (stimulator cells). In culture (over 4 - 6 days), T-cells (responder cells) recognize the foreign class II antigen and undergo transformation (DNA synthesis and enlargement: blastogenesis) and proliferation (mitogenesis). These changes can be recorded by the addition of radioactive (triated, ³H) thymidine into the culture and monitoring its incorporation into DNA. Most modern laboratories, however, are switching to PCR technology for tissue typing using specific probes for MHC specificities.

Generation of cytotoxic T lymphocytes

Another consequence of the MHC antigen and T cell interaction is the induction of cytotoxic T-lymphocytes. When T-lymphocytes are cultured in the presence of allogeneic lymphocytes, in addition to undergoing mitosis (MLR), they also become cytotoxic to cells of the type that stimulated MLR. Thus, T-lymphocytes of 'x' haplotype cultured over 5 - 7 days with B lymphocytes of 'y' haplotype will undergo mitosis and the surviving T-lymphocytes become cytotoxic to cells of the 'y' haplotype. The induction of mitosis in MLR requires disparity of only class II antigens whereas the induction of cytotoxic T-lymphocytes (CTL) requires disparity of both class I and class II antigens. However, once cytotoxic cells have been induced, the effector cytotoxic cells recognize only class I antigens to cause cytotoxicity.

ALLOGRAFT REJECTION

The clinical significance of the MHC is realized in organ transplantation. Cells and tissues are routinely transplanted as a treatment for a number of diseases. However, reaction of the host against allo-antigens of the graft (HVG) results in its rejection and is the major obstacle in organ transplantation. The rejection time of a graft may vary with the antigenic nature of the graft and the immune status of the host and is determined by the immune mechanisms involved.

Hyper-acute rejection

This occurs in instances when the recipient has preformed high titer antibodies. A graft may show signs of rejection within minutes to hours due to immediate reaction of antibodies and complement.

Accelerated (2nd set; secondary) rejection

Transplantation of a second graft, which shares a significant number of antigenic determinants with the first one, results in a rapid (2 - 5 days) rejection. It is due to presence of T-lymphocytes sensitized during the first graft rejection. Accelerated rejection is mediated by immediate production of lymphokines, activation of monocytes and macrophages, and induction of cytotoxic lymphocytes.

Table 1. Different patterns of graft rejection		
Type of rejection	Time taken	Cause
Hyper-acute	Minutes-hours	Preformed anti-donor antibodies and complement.
Accelerated	Days	Reactivation of sensitized T cells
Acute	Days - weeks	Primary activation of T cells
Chronic	Months - years	Causes unclear: antibodies, immune complexes, slow cellular reactions, recurrence of disease.

Acute (1st set; primary) rejection

The normal reaction that follows the first grafting of a foreign transplant takes 1 - 3 weeks. This is known as acute rejection and is mediated by T lymphocytes sensitized to class I and class II antigens of the allograft, elicitation of lymphokines and activation of monocytes and macrophages.

Chronic rejection

Some grafts may survive for months or even years, but suddenly exhibit symptoms of rejection. This is referred to as chronic rejection, the mechanism of which is not entirely clear.

PROCEDURES TO ENHANCE GRAFT SURVIVAL

In clinical practice, the most successful transplantation programs have been with kidneys and corneas. However, other organs are being transplanted with increasing frequency. The success in these programs has been due to a better understanding of immunological mechanisms, definition of MHC antigens and development of more effective immunosuppressive agents.

Strategies for bone marrow transplantation

In bone marrow transplantation, the most crucial factor in donor selection is class II MHC compatibility. Once again an identical twin is the ideal donor. From poorly matched grafts, T lymphocytes can be removed using monoclonal antibodies. The recipient must be immunosuppressed. Malignant cells must be eliminated from the recipient blood (in case of blood-borne malignancies). Methotrexate, cyclosporin and prednisone are often used to control GVH disease.

Other grafts

Corneal grafts do not contain D region antigens and consequently survival is frequent. Small grafts are better and corticosteroids are helpful.

Skin allografts have a very poor success rate and immunosuppressive therapy is relatively ineffective. Nevertheless, they are often used to provide a temporary covering to promote healing in severe skin damage. Indeed, there will be no rejection if the host and donor are perfectly matched (identical twins) or the recipient is tolerant to the donor MHC antigens (bone marrow chimeras).

Disease	Associated Alleles	Frequency in		Relative Risk
		Patients	Control	
Ankylosing spondylitis	B27	90	9	87.4
Reiter's disease (syndrome)	B27	79	9	37.0
Acute anterior uveitis	B27	52	9	10.4
Psoriasis vulgaris	Cw6	87	33	13.3
Dermatitis herpetiformis	DR3	85	26	15.4
Cardiac Disease	DR3	79	26	10.8
Insulin-dependent diabetes mellitus	DR3/4	91	57	7.9

Table 2. Examples of selected immunosuppressive agents

agent	possible mode of action	application(s)
corticosteroids, prednisone	anti-inflammatory, altering T-cell and PMN traffic	organ transplant, hypersensitivity, autoimmune diseases
cyclosporin, FK-506	inhibition of IL-2 synthesis	organ transplant
rapamycin	blocking of IL2-IL2R signal	organ transplant
azathioprine, 6-MP	purine metabolism	organ transplant, autoimmunity
methotrexate	folate metabolism	organ transplant, autoimmunity
cyclophosphamide, melphalan	alkylation of DNA, RNA and proteins	organ transplant, autoimmunity

MHC association with diseases

A number of diseases have been found to occur at a higher frequency in individuals with certain MHC haplotypes. Most prominent among these are ankylosing spondylitis (B27), celiac disease (DR3) and Reiter's syndrome (B27). Other diseases associated with different specificities of the MHC are listed in Table 3. No definite reason is known for this association. However, several hypotheses have been proposed: antigenic similarity between pathogens and MHC, antigenic hypo- and hyper-responsiveness controlled by the class II genes are included among them.

You have learned about

The role of MHC in host-versus-graft (HGV) and graft-versus-host (GVH) disease.

Genetics of the two MHC molecules.

The role of polymorphism and crossover in heterogeneity of MHC antigens in a population.

Methods for detecting MHC antigens (tissue typing).

Immune mechanisms in transplant rejection.

Strategies for successful transplantation.

The ABO Blood Group System

The Early History of Transfusion before Blood Groups

It was recorded in the Bible that the "life of the flesh was in the blood". So from ancient time blood has been intimately associated with life. The most obvious proof of this to the ancients was acute blood loss due to injury or weapons.

The major ancient civilizations of Greece and Rome deduced from their many wars that acute blood loss was one of the most rapidly lethal consequences of any wound. As a consequence one of the main goals was to stop blood loss. The **ancient Greeks** were the first recorded to attempt this by the process of using a tourniquet. This involved placing a tourniquet, on a

limb, above the wound. The aim was to allow the body to form a stable clot and thereby stop bleeding. The major problem was that if left on too long, the tourniquet would deprive the tissue below it of oxygen and gangrene would set in.

It was left to the **Romans** to refine medical treatments of acute blood loss to include not only tourniquets but ligature (the tying off of a blood vessel). The Romans also invented the first surgical clamp that was able to tie off a severed artery while still allowing blood to flow to the limb thereby reducing the risk of fatal gangrene. It is interesting to note that after the fall of Rome these same skills were not rediscovered till the seventeenth century.

Ancients only devised ways of stopping acute blood loss. The first suggested case of transfusion was rumoured to have been given to Pope Innocent VIII in **July 1492**. It was widely believed in the middle age that the drinking of human blood was a method where a person's health could be restored. This should be viewed in the context that medieval "medicine" also considered swallowing paper with the Lords prayer or ground bones of saints and bleeding various parts of the body according to the phases of the moon valid and beneficial therapies. In the case of Pope Innocent VIII a Jewish physician **Abraham Myere of Balmes** suggested sucking the blood of a youth for a restorative tonic for the Pope. As a result three "volunteer" youths were said to have donated a restorative tonic for the Pope and were paid one ducat each. Whether the Pope drank it or it was transfused (unlikely as the circulatory system was not discovered until 1628 by William Harvey) is dubious as it was not reported by any court ambassadors of the time. As for the three young donors, they died of blood loss but had the consolation of being considered martyrs.

The first truly verified accounts of transfusion occurred in the mid seventeenth century. The first transfusions were animal to animal. In **1665 John Wilkins** was the first person, via a syringe, to intravenously transfuse two ounces of blood from one dog to another with no harmful effect. These experiments were extended by **Richard Lower** who in **1666** continued transfusion experimentation in dogs with vein to vein and artery to vein transfusions. He discovered that artery to vein transfusions worked due to the higher arterial blood pressure (4).

The first human involvement as a transfusion recipient was performed by a Frenchman **Jean Denys on the 25 of June 1667**. The "scientific" principle behind the use of animals blood was that animals possessed purer blood than humans as they were not prone to the vices of drinking and eating that humans were (4). To this end a transfusion was used to treat Antoine Mauroy, a newly wed, who was prone to run away to Paris for extended bouts of debauchery and vice. To cure this Jean Denys selected calfs blood due to the animals gentle nature. To consolidate the treatment a second transfusion was performed a week later. One small problem is the immune system is very efficient, and antibodies to the calf cells would have formed. This became immediately evident when Mauroy complained of kidney pain and a heavy sensation in the chest and passing of dark urine. This was undoubtedly the first recorded case of an acute haemolytic transfusion reaction. Later attempts to repeat the treatment were refused by the patient (1).



Fig1 Animal to human

transfusion 1667

The practice of transfusion remained at this level until the appearance of **James Blundell** in the early 19th Century. James Blundell was motivated by the consequences of post partum haemorrhages who often went into shock and died from acute blood loss. Unlike earlier attempts he refused to use animal blood based on work by **Dr John Leacock** whose experiments showed the blood of one species may be harmful to another (1). In **1818 Blundell** transfused 12 - 14 ounces of blood via a brass syringe into a patient who temporarily improved but died of their initial disease(1). However the practice of transfusions from animals did not disappear with papers as late as **1874 Franz Gesellius** and **Oscar Hasse advocated the use of lambs blood**.



The major problem before the discovery of anti coagulants was that blood collected for transfusion clotted. To get around this two methods were devised. The first was by **Jean Prevost** and Jean Baptiste Dumas who found that stirring collected blood caused the creation of fibrin and preventing the remaining blood from clotting. They also found that defibrinated blood was just as good as untreated blood for resuscitation (1). The second method invented by Gesellius involved capillary transfusions via a device (fig 2) which simultaneously punctured the skin in many places. The blood was then sucked into a bowl and transfused. The end of animals being used as donors was accomplished in **May 1874** by **Ponfick** and **Landois** who studied the death of a 34 year old woman transfused by a sheep and noticed lysed red cells in her serum and haemoglobinuria. This problem of red cell lysis was noted when blood was transfused between

species (1).

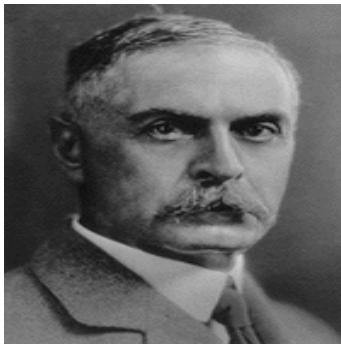
**Fig 2 . Gesellius' capillary transfusion
(Transfusion 1997; 37 : pg 555)**

Once the use of animals ceased there was the major problem of deaths resulting even when human blood was used. This was due to the major barrier posed by the **ABO blood group system**. By pure random chance based upon caucasian frequencies of the ABO blood groups the chance of a compatible transfusion is 64.4 % (1). The evolution of safer transfusions that we know today was heralded by the **discovery of ABO blood groups by Karl Landsteiner** in 1901.

The Discovery of ABO Blood Groups

As has been briefly examined, transfusion success was a very hit and miss affair even when humans were used as blood donors. This was directly due to the major barrier posed by the ABO blood group system. The consequences of an ABO incompatible blood donation is acute and potentially lethal intravascular destruction of the transfused red cells.

The ABO blood group system was discovered by **Karl Landsteiner** who was born in Vienna on the 14 / 06 / 1868. He studied medicine at the University of Vienna , graduating in 1891.



In 1896 he became an assistant to Max von Gruber in the Hygiene Institute of Vienna and became interested in the mechanisms of immunity and the nature of antibodies. In 1898 he became an assistant in the university Department of Pathological Anatomy in Vienna (5). It was during this time he started to investigate whether differences existed between different peoples red cells. This was inspired by work done by **Landois and Ponfick** who in **1874** discovered that transfused red cells from one species to another and some humans to other humans lysed in the circulation. Death was attributed to the organ damage and hyperkalemia from the rapid release of potassium from the lysed red cells (1).

Fig 1. Karl Landsteiner

To investigate for any potential differences between human red blood cells **Landsteiner in 1901** chose a simple experiment. He mixed the serum and red cells from different people and observed the reaction. As a result of observing the agglutination patterns he described three distinct groups whose serum possessed naturally occurring antibodies which could react with some other peoples red cells. As a result Landsteiner divided these individuals into three groups called groups A, group B and group C. The group C was later changed to group O (1, 6, 7). In **1902** the fourth group of the ABO system was defined by **Decastello and Sturli** who identified people whose serum did not naturally produce antibodies that agglutinated other human red cells. This group was called AB.

Summary Of Early Experiments on the nature of the ABO blood Groups

- **Group A** - Possessed **A antigen** on their red cells and had naturally occurring **antibodies (anti-B) that reacted with group B and AB people**. Their red cells were agglutinated by group O and B people.
- **Group B** - Possessed **B antigen** on their red cells and had naturally occurring **antibodies (anti-A) that reacted with group A and AB people**. Their red cells were agglutinated by group O and A people.
- **Group AB** - Possessed both **A and B antigens** on their red cells and **produced no** naturally occurring **antibodies**. Their serum reacted with no other ABO group individuals. Their cells agglutinated with the sera from all other groups.
- **Group O** - Had **neither A or B antigens** on their red cells and had naturally occurring **antibodies (anti- A and anti - B)** that agglutinated all red cells from group A, B and AB people. Their red cells were not agglutinated by serum from any ABO group (1, 7, 8).

These discoveries breached the potentially fatal barrier the ABO groups posed for human to human transfusions. The use of these discoveries in "typing" human donors and transfusion recipients received little attention until they were published in a book in 1909 defining the ABO blood groups. It also stated that red cells were not destroyed when people received transfusions from people of the same ABO blood group (5). The **inheritance of the ABO blood groups** was later suggested in 1910 (5). Dr Landsteiner died on 24 of June 1943 in his laboratory from a heart attack.

In experimental studies in **1911**, **Von Dunergern** and **Hirschfeld** discovered that **primates also possessed similar A and B like antigens** (10). The investigation and study of these similar antigens was subsequently undertaken in **1925** by

Landsteiner and Miller(10). It was proposed that the sharing of the ABO system was due to **shared genetic evolution** between humans and primates.

These discoveries allowed the creation of the first transfusion service in the world founded by the British Red Cross in **1926**. The Australian Blood Bank was founded by **Dr Lucy Bryce**, with the first blood transfusion service operating at the Royal Melbourne Hospital in **1929**.

Inheritance and Genetics

The fact that the ABO blood group system was inherited was suggested in **1910** by **Epstein** and **Ottenberg**. The confirmation of the ABO system being genetically inherited was by **von Dungern** and **Hiszfeld**, who studied 72 families with 102 children. They found that the inheritance of the A and B agglutinogens obeyed **Mendel's laws**.

They discovered that the **ABO gene was autosomal** (the gene was not on either sex chromosome). Therefore each person has two copies of genes coding for their ABO blood group (one maternal and one paternal in origin). It was observed that the **A and B blood groups were dominant over the O blood group**. It was also found that the **A and B group genes were co-dominant**. This meant that if a person **inherited one A group gene and one B group gene** their red cells would **possess both the A and B blood group antigens**. These alleles were termed A (which produced the A antigen), B (with produced the B antigen) and O (which was "non functional" and produced no A or B antigen) (1, 7, 8).

Table 1: Summary of Blood group, Red cell antigens and the possible inherited genotype.

Human Blood Group	Red Cell Antigens	Serum Antibodies	Possible Genotype
A	A antigen	Anti-B	AA or AO
B	B antigen	Anti-A	BB or BO
O	None	Anti A and Anti-B	OO only
AB	A and B antigen	None	AB only

Fig 1: A sample family pedigree of three generations demonstrating the basic Mendelian Inheritance of ABO blood groups.

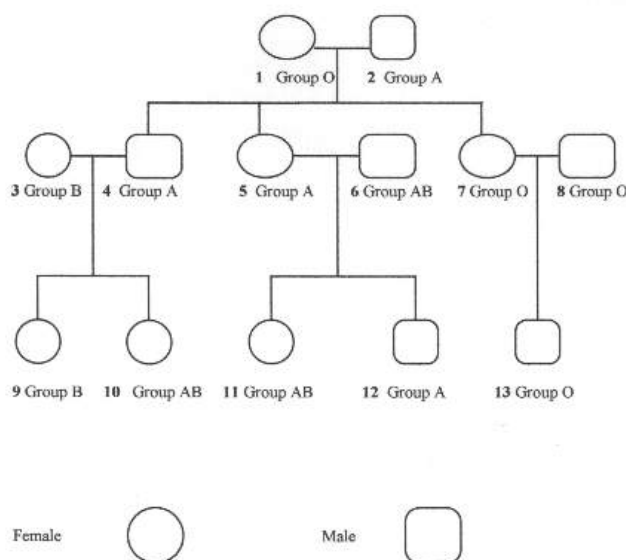


Table 2: Genotypes Of The Individuals In Figure 1

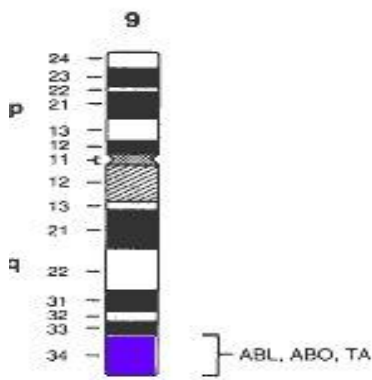
Pedigree ID number	Genotype	Reason Why
1	OO	As the O allele is recessive to both the A and B alleles to express the O blood group phenotype only the O gene can be present.
2	AO	As this male has one child who is blood group O (#7) he must be a heterozygote. If he was homozygote the offspring must have inherited an A gene. If this were present as it is dominant to the O gene the offspring would have been blood group A.
3	BB or BO	As the B gene is dominant to the O gene this individual could possess one or two copies of the B gene. If this person and their partner #4 had an O child then we could safely conclude that this person must have a BO genotype. Based on their offspring not enough information exists to clarify their phenotype.
4	AO	Due to Mendel's laws this person must have received one maternal and one paternal ABO allele. The mother was homozygous for the O gene so this individual must have one O allele. To express the A phenotype they require one A allele which is dominant to the O allele, which they inherited from individual #2.
5	AO	As for individual #4
6	AB	This individual produces both the A and B antigens. They must therefore have one A gene and one B gene. These are co-dominant, hence both alleles are expressed in the phenotype.
7	OO	As for individual #1
8	OO	As for individual #1
9	BO	This individual could not have inherited the A gene from their father (#4) as it would be expressed along with the maternal B gene and would have produced an AB phenotype. They must have received their father's O gene which is recessive to the inherited maternal B gene. Based on Mendelian Genetics the other potential groups for this person from parents 3 and 4 could have been A (AO), AB or O.
10	AB	As for individual #6
11	AB	As for individual #6
12	AA or AO	This person could be AA if they inherited the A gene from both their mother (#5) and their father (#6). As this individual's mother is an AO heterozygote it is possible that they inherited an O gene. This would mean that the A gene was of paternal origin. As the A gene is dominant they would express the A blood group.
13	OO	As for individual #1

Allelic polymorphisms of the ABO system

The ABO genes

The normal human has 46 chromosomes in their genotype. This consists of 46 chromosomes that are divided into 22 homologous paired autosomal chromosomes (one of maternal, the other paternal in origin) and two sex determining chromosomes (X and Y). These chromosomes contain all the genetic blueprints for the creation and maintenance of human life. The genetic blueprint consists of about three billion bases of Deoxyribonucleic Acid (DNA) consisting of four different bases; adenine (A), thymine (T), guanine (G) and cytosine (C) and protein. This DNA exists in the cell nucleus as a highly condensed strand (20, 21). The DNA in the nucleus consists of three billion bases, of this only about 10-15% are

actually incorporated into genes. Each chromosome contains a few thousand genes. It is the gene that is responsible for the coding of a single trait (i.e. enzyme, protein, etc.) (20, 21).



The ABO blood group phenotype is actually determined by alleles on two chromosomes. These are **chromosome 9** which possesses the ABO gene allele and **chromosome 19** which possess a gene that codes for the creation of the substrate antigen (**H antigen**) which then may or may not be altered by the allele (s) present on chromosome 9. Unlike many other red cell antigens both these genes **do not code for the actual antigen**. These **genes code for enzymes** that actually create the antigen on a precursor structure on the red cell. These **enzymes determine a persons ABO blood group** by catalysing the attachment of a blood group defining sugar onto a basic carbohydrate precursor antigen. The enzyme produced by the gene on **chromosome 19 adds a sugar** to the precursor carbohydrate to **produce the H antigen**. The ABO gene is responsible for determining if a person is an A, B or O phenotype.

Fig 1: The ABO Gene

The **A and B gene** each code for an **active enzyme that recognize the H antigen** and attach a group defining sugar. The **O gene** is actually an **amorph** gene meaning that it **does not produce an active enzyme**. As a result blood group O people are not able to modify the H antigen which remains the only ABO antigen expressed on their cells.

The **ABO gene locus** is located on the **long arm of chromosome 9 (9q34)**. Except in rare ABO groups such as the **Bombay blood group** it is the alleles at this locus that defines the ABO phenotype. The ABO alleles consist of a 30 kb nucleotide which consists both exons and introns. The exon is a discontinuous nucleotide sequence that codes for the final amino acid sequence. The final coding sequence of the gene product consists of these exons being spliced together after all superfluous intervening sequences are removed (introns). The final **coding region of the gene** consists a **1062 bp nucleotide** sequence **consisting of 7 exons**. This produces an **active enzyme** that is **353 amino acid residues** in length (25, 28).

The **first 5 exons** are relatively **small**. The **sixth exon** is a 135 nucleotides and **codes for the C terminal catalytic domain of the enzyme**. The largest exon is the **seventh exon** which consists of 688 nucleotides. It is the seventh exon which **codes for the different transferase enzyme specificity** produced by the **A and B alleles** (25).

Differences Between the A and B Alleles

The structure of the DNA coding sequence and the enzyme produced by transcription of the ABO genes show a **high degree of similarity between the A and the B blood group gene**. The differences between the two most common forms of these two alleles results from a series of **point mutations**. The result of these mutations are **four amino acid substitutions in the enzyme at positions 176, 235, 266 and 268** (25, 26, 28, 29). These mutations result in a conformational change in the enzyme and , subsequently, the observed functional specificity (25). In particular the residue at **position 268 is critical for A or B transferase activity**. The **A subgroups** and **B subgroups** result from different mutations in the coding DNA and result in different transferase activity in the subsequent enzyme (25, 29).

Table 1: Defining Amino Acid Differences between the enzymes Produced by the Common A and B Blood Group Alleles (25)

	Residue 176	Residue 235	Residue 266	Residue 268
A Group Allele	Arginine	Glycine	Leucine	Glycine
B Group Allele	Glycine	Serine	Methionine	Alanine

Genetics of the O Blood Group

The **O blood group** results from the gene at the **ABO locus on chromosome 9** producing a **non functional transferase enzyme** (8, 25, 26, 28, 29). This non functional transferase will not recognize the basic H antigen and is subsequently unable to add a blood group defining sugar onto the H antigen. As a result only the H antigen will be present on an O person's cells (8, 25, 26, 29).

Initially the O gene was found to be almost identical in the DNA sequence to the A allele. The only difference observed was a **point mutation at position 258 (sixth exon)** in the coding region close to the N terminal. This **caused a frame shift** leading to a **completely different protein of only 116 amino acids with no transferase activity** (25, 26, 28). This has been called the **O¹ variant**.

A **second mutation** producing the O phenotype has been identified **involving residue 268**. The A and B transferases have small non polar residues in this position. In the case of the O mutation a point mutation occurs that **substitutes arginine which is large with a basic side chain**. This **causes a radical conformational change** in the transferase enzyme rendering it inactive. This has been called the **O² variant** (25, 28).

The **third mutation** identified has **two different mutations**. First a **deletion** of nucleotide at **position 1060** which **alters the stop codon**. This causes a longer protein to be produced. There is also a **nucleotide insertion between position 798 and 804**. This produces a non functional transferase called the **O³ variant** (25).

Table 2: Summary of the O allele Polymorphism's of the ABO gene (25, 30)

O Variant	Mutation observed
O ¹ Variant	Point mutation at nucleotide 268 causing a truncated 116 Amino Acid protein.
O ² Variant	Point mutation causing a Amino Acid Substitution at position 268 producing a conformational change in enzyme shape.
O ³ Variant	Two mutations: a) Deletion of nucleotide 1060 which alters the stop codon. producing a longer protein. b) Nucleotide insertion between position 798 and 804

Synthesis and Structure of ABO Blood Groups

The **ABO antigen system is a ubiquitous antigen system**. This means that the A, B or O (H antigen) is not limited only to an individual's red cells. The ABO group of an individual is expressed on diverse cells and tissues. In certain individuals who possess a specific gene, the ABO antigen is also expressed in saliva and other body fluids (secretors) (8, 25, 33).

The successful creation of the final **ABO blood group is however the final stage of a complex series of enzymatic actions** with approximately 100 glycosyl transferases being involved in the creation of the carbohydrate oligosaccharides to which the H, A or B antigen is added (8).

The **A, B or O blood group** of an individual involves the **interaction** between **enzymes** which are the product of **two genes**;

- The H gene on chromosome 19 codes for a fucosyl transferase that synthesizes the H antigen.
- While the gene defining the A, B or a non functional enzyme is located at ABO gene locus on **chromosome 9** (8, 25, 27, 30, 33).

The **ABO antigen** expressed by an individual's cells are **carbohydrate antigens**. These are **constructed of oligosaccharides chains constructed in a stepwise manner** with each sugar being added to the growing chain by a specific enzyme (**glycosyl transferase**).

These oligosaccharides are **attached to different types of precursor chains such as polypeptides or ceramide** which act as transmembrane anchors (8, 25, 26, 33).

The synthesis of an ABO antigen is a sequential 2 step process involving:

- **H Antigen production**
- **A Antigen and / or B Antigen production**

H Antigen Precursor

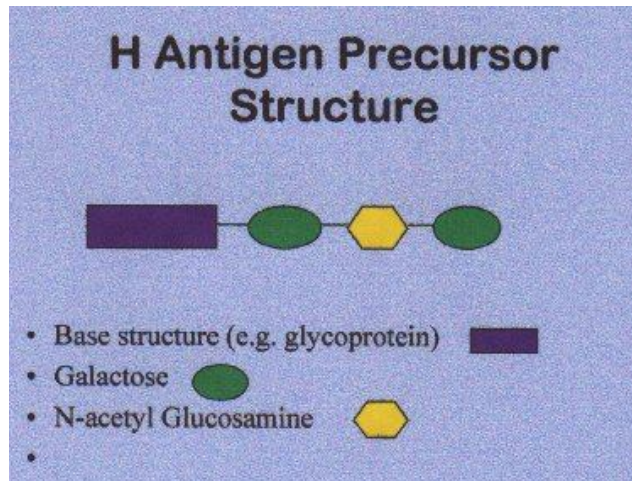


Fig 1: H Antigen Precursor.

The building block for the construction of the A,B or H antigen is a precursor oligosaccharide which can vary from a few sugar molecules in a simple linear chain to more complex structures (33). The oligosaccharide is constructed in a sequential manner by the enzymatic addition of sugars to the terminal end of the oligosaccharide chain, this is then attached to a membrane anchor which may be a glycoprotein, glycolipid, etc. No matter the molecule that the molecule is attached to the last three sugars on the chain are; galactose, N acetyl glucose and a terminal galactose (8, 25, 26, 33).

Step 1: H Antigen Synthesis

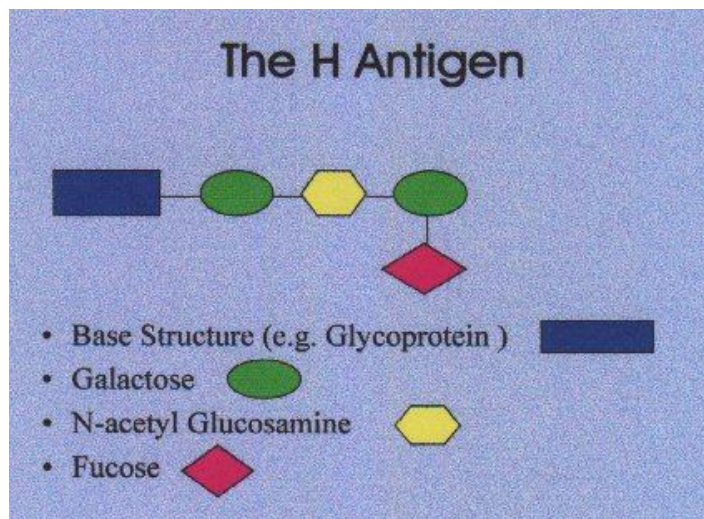


Fig 2: H antigen

The Synthesis of the H antigen is **controlled by the H gene on Chromosome 19**. This gene **codes for the synthesis of an enzyme** called **fu cosyl transferase (FUT 1)**. This enzyme recognizes the terminal galactose of the precursor oligosaccharide and **catalyses the addition of fucose to the terminal galactose**. This enzymatic addition creates the **H antigen** which is the antigen **expressed on group O individuals** and is used as the precursor **A and B group antigens** (8, 25, 26, 33).

Step 2: Synthesis of the A and B antigens

The A Antigen :

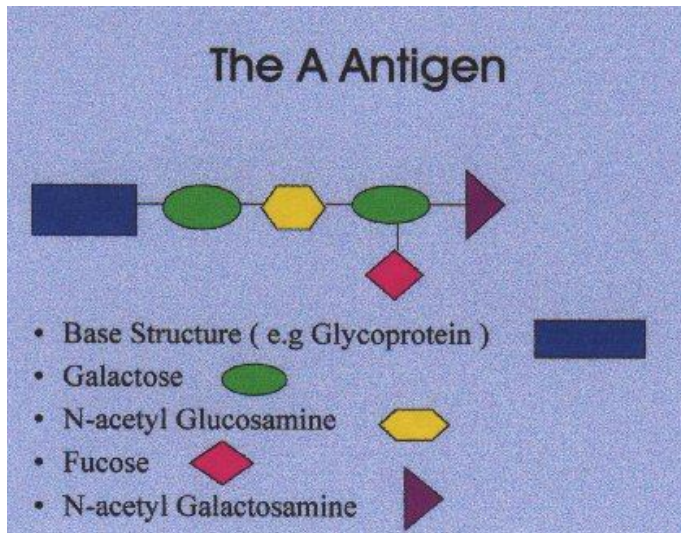


Fig 3: The A antigen.

The A antigen is produced via the action of an enzyme (a glycosyl transferase) coded on chromosome 9 at the ABO gene locus. This enzyme uses the H antigen as a substrate and **catalyses the addition of the sugar N acetyl galactosamine** to the terminal galactose to produce the A antigen. **The A enzyme can only produce the A antigen if a H antigen is already present** (8, 25, 26, 30, 33).

The B Antigen :

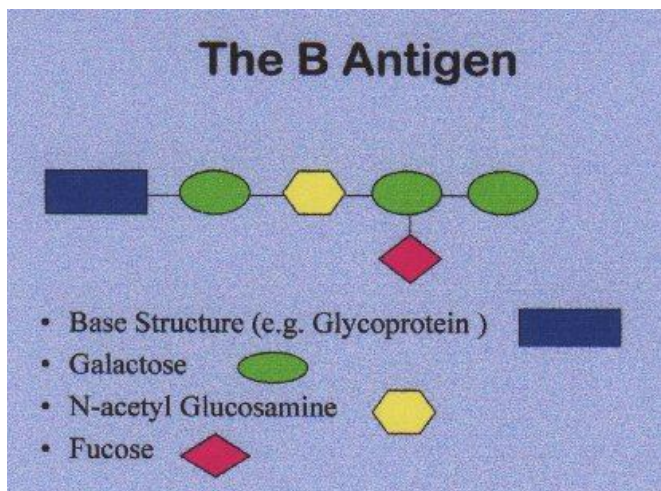


Fig 4: The B antigen

The B antigen is produced by an enzyme coded for by a gene at the ABO locus on chromosome 9. The enzyme (a glycosyl transferase) uses the H antigen as a substrate and catalyses the addition of the sugar galactose to the terminal galactose of the H antigen. This produces the B antigen. The B enzyme can ONLY produce the B antigen if a H antigen is present.

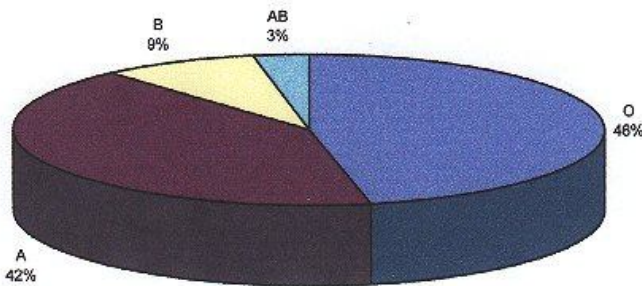
Frequencies of the ABO blood groups

Home

The frequencies of the ABO blood group system vary significantly in various populations and ethnic groups around the world. There have been several reasons given for this phenomenon such as **population migration** and **diseases** where certain blood groups were thought to provide a selective advantage or disadvantage.

In the case of migration, **ethnic communities** that experienced **little migration** due to their location being remote maintain a **high degree of genetic similarity and relatively low ABO group polymorphism**. An example of this situation is the **Aboriginal population** of Queensland Australia which has approximately **over 75%** of the Population being **O Positive** (8). This can be compared to the frequencies of blood groups observed in the population of Britain which showed only 46% of people are of the O phenotype (Figure 1). This can be explained by the historical observation that Britain has been settled by Celts, Romans, Germanic Saxons, Scandinavian Vikings and Normans (22, 23). As the ABO blood groups are under genetic control, it would be expected that a certain gene would predominate (e.g. the O gene in Queensland Aboriginals) in a particular homogeneous tribal group.

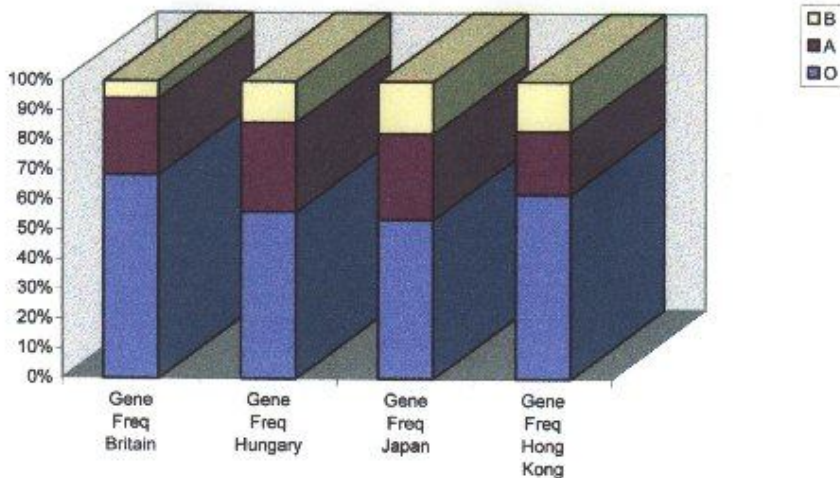
Fig 1: The ABO Phenotype Frequency in Britain in 1946. (8)



As **tribal and ethnic groups migrated and intermarried** the **frequencies** of particular **genes** (and hence phenotypes) **would increase or decrease within the total population**. In a similar way the frequencies of the ABO genes and hence the ABO phenotype would alter if they provided selective advantages or disadvantages. As **different populations are affected by human migration to different extents** and are

exposed to different pathogens the gene frequencies and therefore the subsequent blood group phenotypes will differ from one region / ethnic group to another. The effect of these factors on different gene frequencies is illustrated in figure 2.

Fig 2: A comparison of the ABO Gene Frequency for Four Different Ethnic Populations. (8)(17)(18)(19)



In Australia the frequency of the various ABO phenotypes as illustrated in table 1 is similar to that observed in the study in Britain in 1946 (24). Some subtle frequency differences would reflect differences in ethnic migration frequencies when compared to Britain.

Table 1: The ABO phenotype frequency in Australia (24)	
ABO Phenotype	Frequency (%)
Group O	49
Group A	38
Group B	10
Group AB	3

The frequency of the ABO blood groups are not however as straight forward as these tables and figure indicate. This is because within the blood groups of the ABO system there exist **subgroups of the A phenotype**, **subgroups of the B phenotype** and rare groups such as the **Bombay blood** group.