Biomolecular chemistry

5. Antibodies: structure and function

Required reading: **Sections 5.1 to 5.3** of
Mikkelsen and Cortón, *Bioanalytical Chemistry*

Primary Source Material

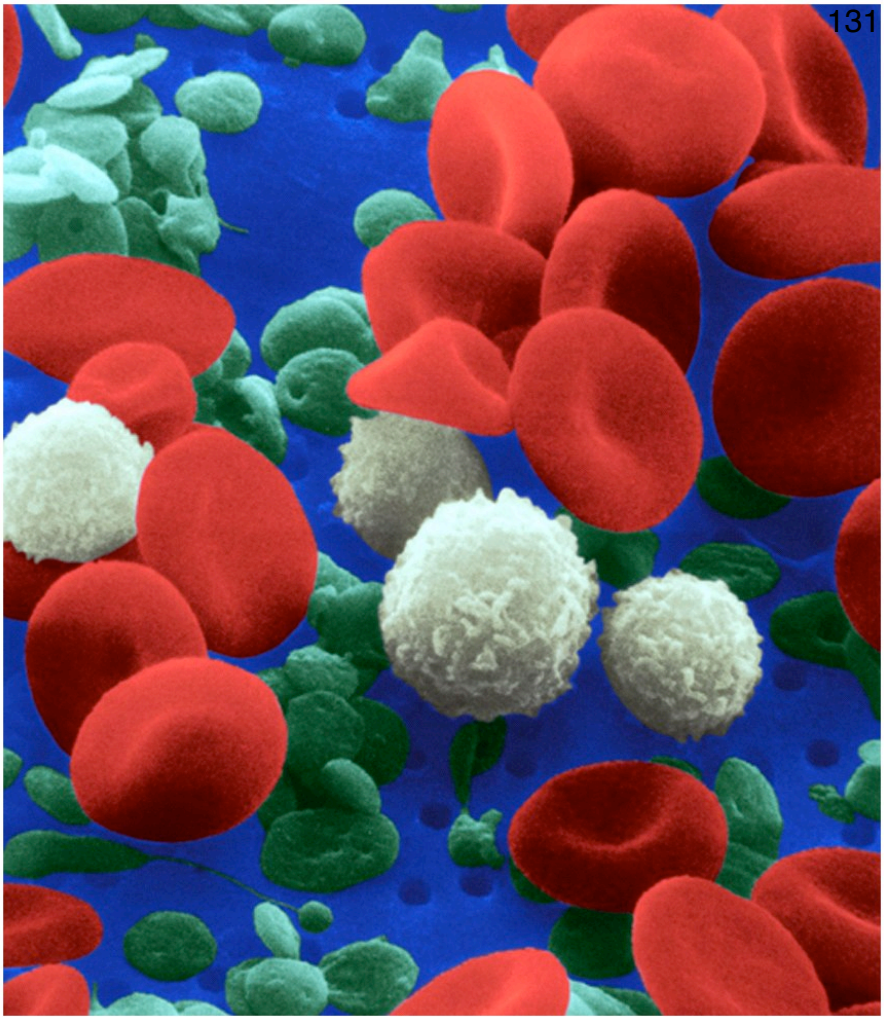
- Biochemistry Chapter 33: Berg, Jeremy M.; Tymoczko, John L.; and Stryer, Lubert (NCBI bookshelf).
- Many figures and the descriptions for the figures are from the educational resources provided at the Protein Data Bank (http://www.pdb.org/)
- Most of these figures and accompanying legends have been written by David S. Goodsell of the Scripps Research Institute and are being used with permission. I highly recommend browsing the Molecule of the Month series at the PDB (http://www.pdb.org/pdb/101/motm_archive.do)

Objectives:

- You will have a very basic understanding of the immune response and how antibodies are generated in humans
- You will have a very good understanding of the structure of immunoglobulin G (IgG).
- You will understand how IgG molecules are able to recognize such a broad range of antigens.
- You will know the difference between polyclonal and monoclonal antibodies
- You will understand the difference between antigen and epitope.
- You will have some appreciation for the impact of protein engineering on antibody technology

Cellular components of blood

- red blood cells
- white blood cells
- platelets
- plasma



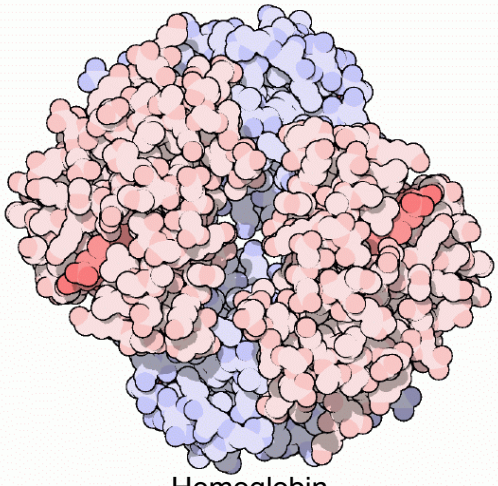
http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookcircSYS.html - Blood

- Mammalian blood consists of plasma and a number of cellular and cell fragment components.
- Plasma: The liquid part of the blood, which makes up about half of its volume. Blood plasma contains antibodies and other proteins. It is taken from donors and made into medications for a variety of blood-related conditions. Plasma has 90% water and 10% dissolved materials including proteins, glucose, ions, hormones, and gases. It acts as a buffer, maintaining pH near 7.4. Note that serum is essentially similar in composition to plasma but lacks fibrinogen and other substances that are used in the coagulation (blood clotting) process.
- Red blood cells, also known as erythrocytes, are flattened, doubly concave cells about 7 μm in diameter that carry oxygen associated in the cell's hemoglobin. Mature erythrocytes lack a nucleus.
- White blood cells, also known as leukocytes, are larger than erythrocytes, have a nucleus, and lack hemoglobin. They function in the cellular immune response.
- Platelets result from cell fragmentation and are involved with clotting.

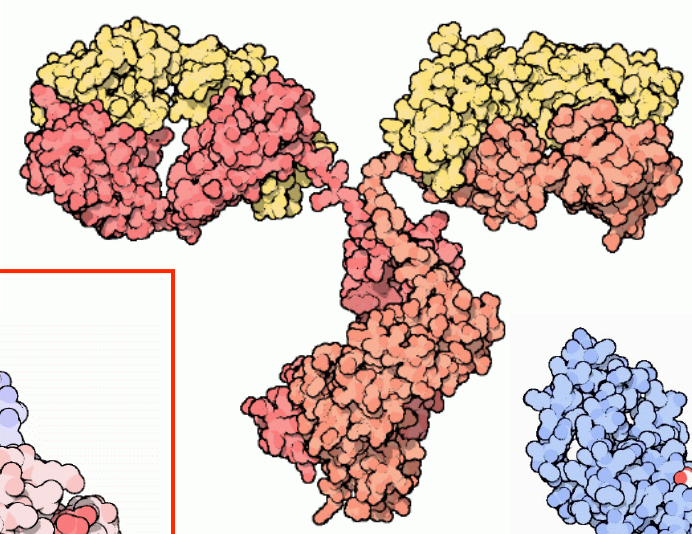
Protein components of blood

Plasma

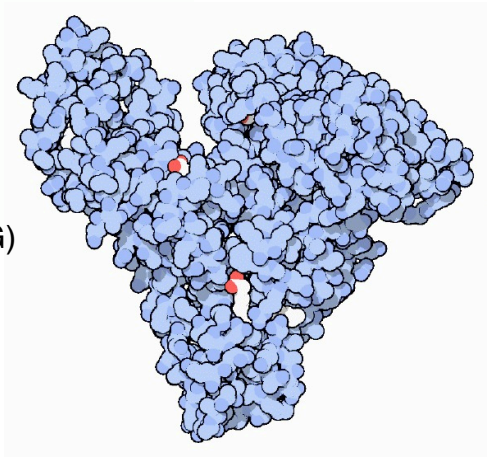
Red blood cell



Hemoglobin



Immunoglobulin G (IgG)

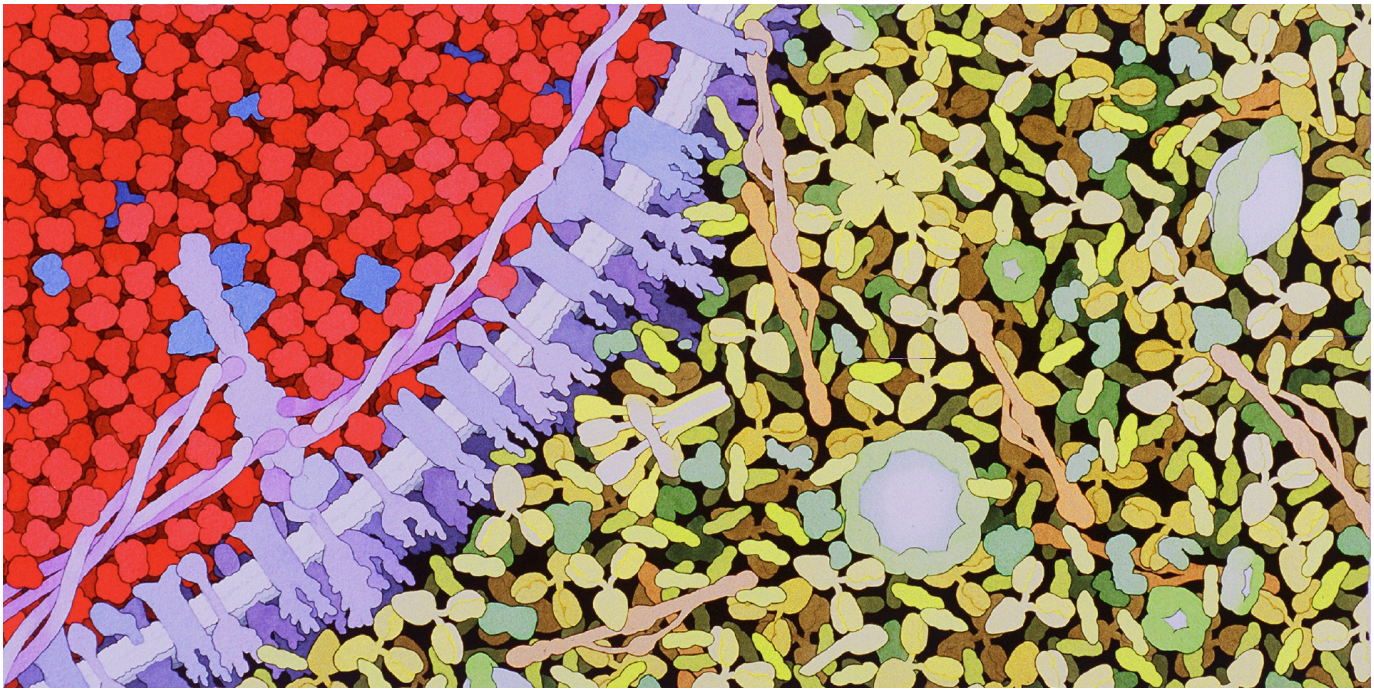


Albumin

http://www.carbonbased.com/cbcblood.htm - Protein and
David S. Goodsell: The Molecule of the Month appearing at the PDB

- Hemoglobin is the main transporter of oxygen and carbon dioxide in the blood. It is composed of globin (a protein) and heme (a cofactor) which contains iron atoms and imparts the red color to hemoglobin. Hemoglobin is densely packaged into red blood cells.
- Albumin is the major constituent of serum protein (usually over 50%). It helps in osmotic pressure regulation, nutrient transport, and waste removal.
- Immunoglobulin G is a member of a class of blood plasma proteins known as globulins. Immunoglobulin is important in the immune response as we will see in the following slides.

The major serum proteins are albumin and immunoglobulin

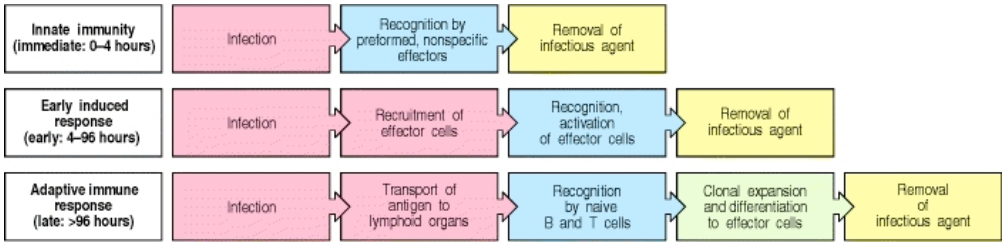


David S. Goodsell: The Molecular Perspective

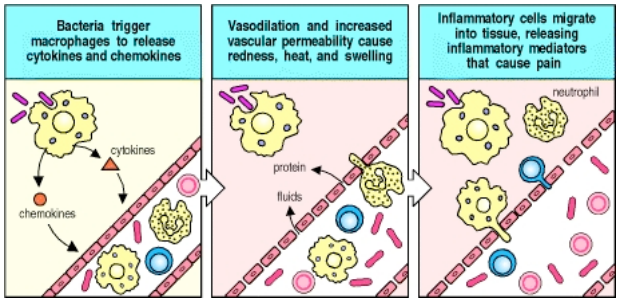
- An artistic/scientific rendition of blood by David S. Goodsell.
- This illustration shows a cross-section through the blood, with blood serum on the right hand side and a red blood cell (RBC) on the left hand side
- Blood serum is filled with antibodies, circulating and searching for foreign molecules. In this illustration, the antibodies are coloured yellow: look for Y-shaped IgG, IgA with two antibodies back-to-back, and IgM with five antibodies in a star.
- Other molecules in this portion of blood serum include stick-like fibrinogen molecules, snaky von Willebrand factor, low density lipoproteins (large circular molecules), and many small albumin proteins.
- The large UFO-shaped objects are low density lipoprotein and the six-armed protein is complement C1.
- The red blood cell is filled with hemoglobin which is shown in red.

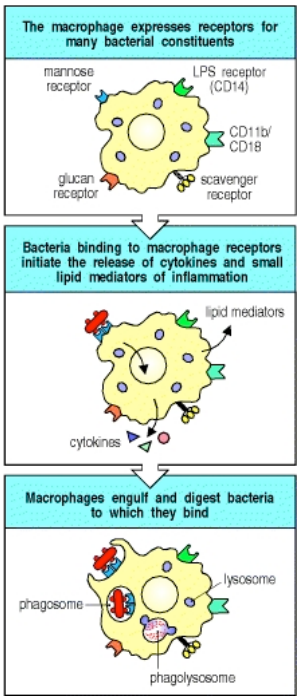
‘Innate’ and ‘adaptive’ immunity system’

- The human immune system recognizes and destroys foreign invaders, which could be molecules (generally proteins), viruses, bacteria, or other microorganisms
- The immune system is divided into two categories: innate and adaptive immunity



Innate immunity is the first response to an infection

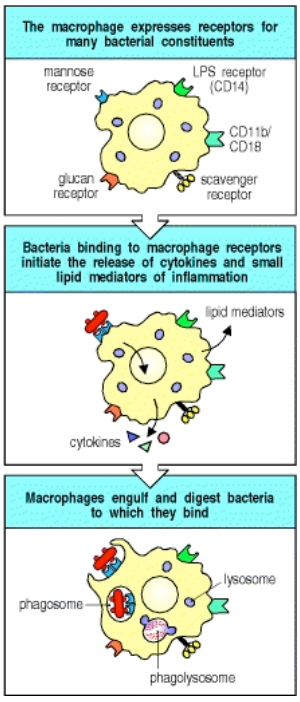


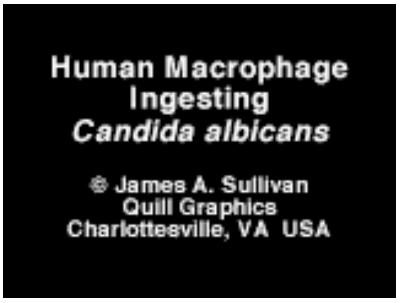


- The immediate innate immunity response is for macrophages (white blood cells) to attack and ingest the foreign invader
- The macrophages also release signalling molecules that cause other ‘defender’ cells to come to the site of the body where the invaders have entered. This is the ‘early induced response’ and is better known as inflammation. As fluid and cells arrive at the site of the infection, the tissue swells, turns red and hot, and becomes painful.

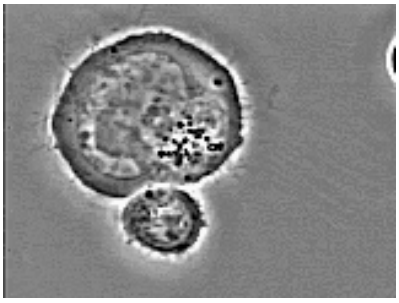
- Images from: Immunobiology: The Immune System in Health and Disease. 5th edition. Janeway CA Jr, Travers P, Walport M, et al. New York: Garland Science; 2001.
- Courtesy of NCBI bookshelf

How does our immune system protect us from foreign molecules, viruses, and bacteria?

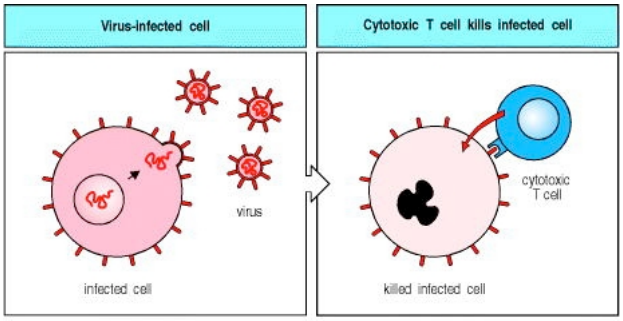




Macrophages can recognize, ingest, and destroy antigens, viruses, bacteria, or other invaders.

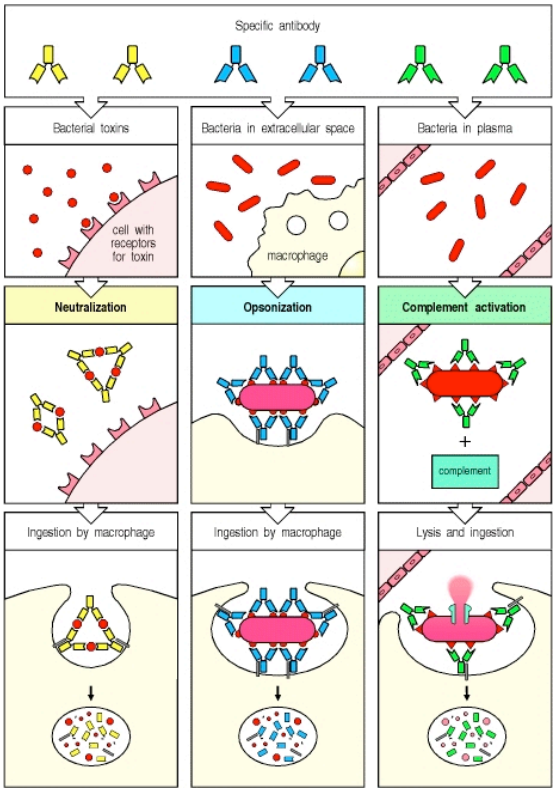


If the macrophage is too late and the bodies own cells have been infected with virus, there are special lymphocytes that can attack and kill the infected cell.



- The presence of foreign molecules on the surface of invadors mark these bacteria (or virus or whatever) for ingestion by phagocytic cells of the immune system. If the antigen happens to be on the surface of a virus or bacteria, the whole virus particle or bacterial cell will be ingested and destroyed
- There are special white blood cells (cytotoxic T lymphocytes) in the blood that will attack the bodies own cells if they have been infected with virus.
- The recognition of these foreign molecules can occur through ‘general’ receptors on the surface of the macrophage or cytotoxic T-cell. This is what would happen as part of the innate response.
- However, invaders can also be marked for destruction through the action of the adaptive immune response. The key to the adaptive immune response is that the antibody molecules bind with high specificity and affinity to the invader.
- Immunobiology: The Immune System in Health and Disease. 5th edition. Janeway CA Jr, Travers P, Walport M, et al. New York: Garland Science; 2001.
- Courtesy of NCBI bookshelf

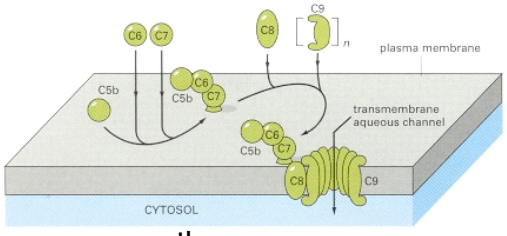
Our adaptive immune system depends on having specific antibodies that bind the invader



Neutralization: by binding to a toxin, an antibody can block its function and make it non-toxic

Opsonization: by coating an invader, antibodies mark it as something to be destroyed, and it will be ingested and destroyed by a macrophage.

Complement activation: antibodies can activate the complement system, which is a set of blood proteins that can destroy an invader directly, and/or make it more likely to be eaten by a macrophage.

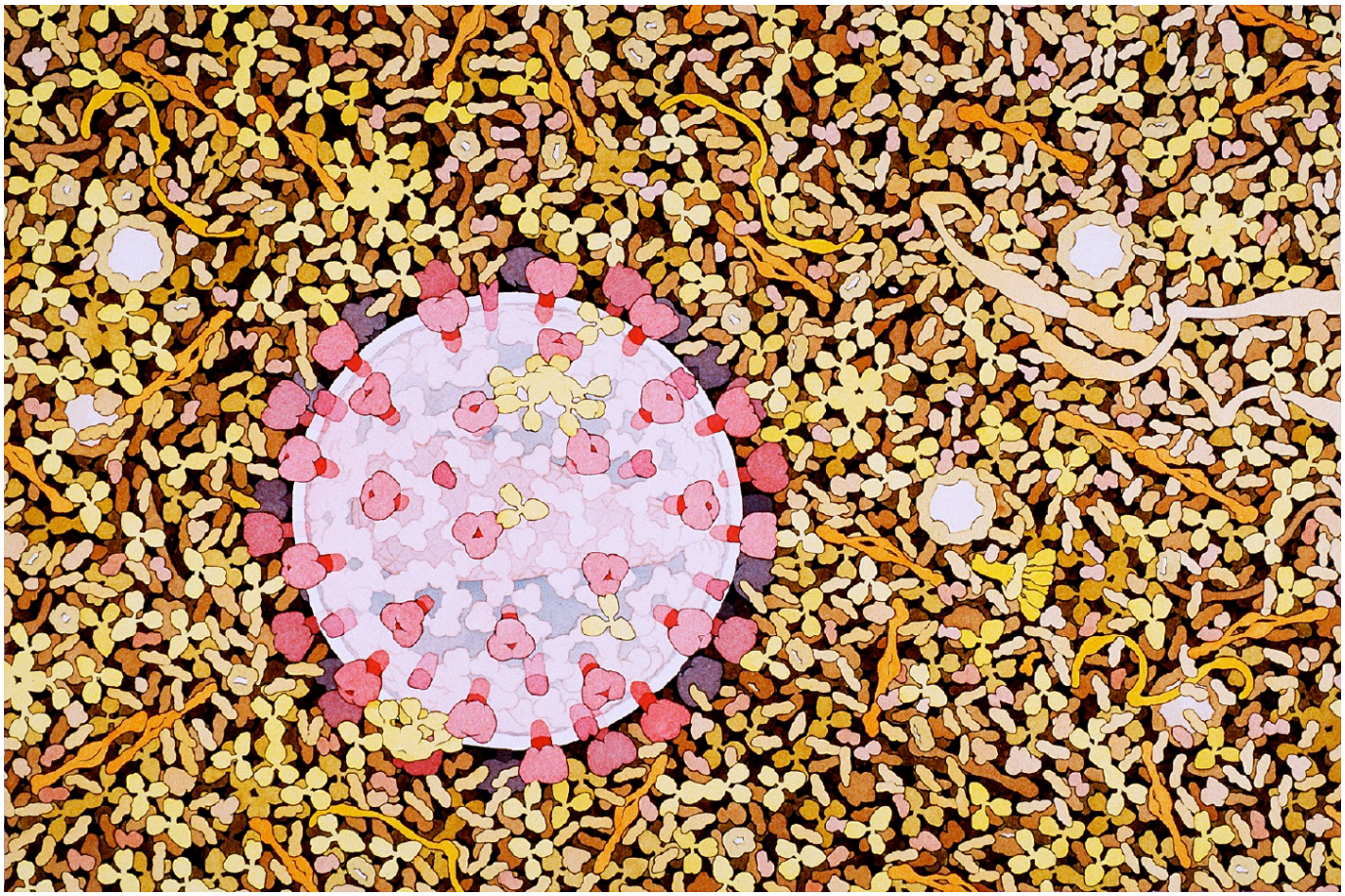


pathogen

- The rest of this section will focus on antibodies.
- It is important to keep in mind that immunology is a huge subject that could be argued to rival all of chemistry in terms of its complexity and the size of the body of knowledge.
- Accordingly, we will be taking a highly simplified view of how the immune system works.

- Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002.
- Immunobiology: The Immune System in Health and Disease. 5th edition. Janeway CA Jr, Travers P, Walport M, et al. New York: Garland Science; 2001.
- Courtesy of NCBI bookshelf

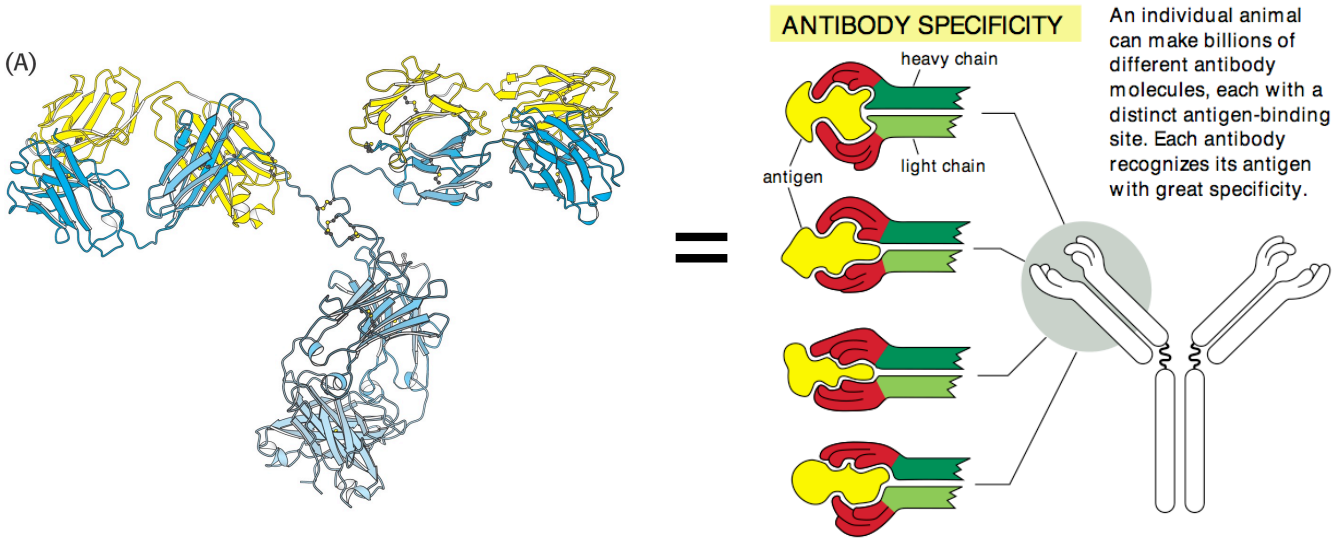
Antibodies ‘attack’ invaders in our blood



David S. Goodsell: http://www.scripps.edu/pub/goodsell/

- In this illustration, an HIV surface protein is the antigen that is inducing the immune response. It is under attack by the immune system. An antigen is a substance capable of inducing a specific immune response. The term ‘antigen’ is derived from the generation of antibodies to such substances.
- Often antigens are foreign proteins (or parts of them) that enter the body via an infection. Sometimes, however, the body's own proteins, expressed in an inappropriate manner (where or when they are not usually seen), are treated like antigens by the immune system.
- It is important to recognize that bacteria or viruses are not themselves antigens but they contain antigens both on their surface and inside them. Such antigens can be isolated and used to safely vaccinate against infection with the whole organism.
- The immune response is a very complex process and we will be taking a greatly simplified view in this course.

Humans have billions of different antibody molecules, each with a unique binding site



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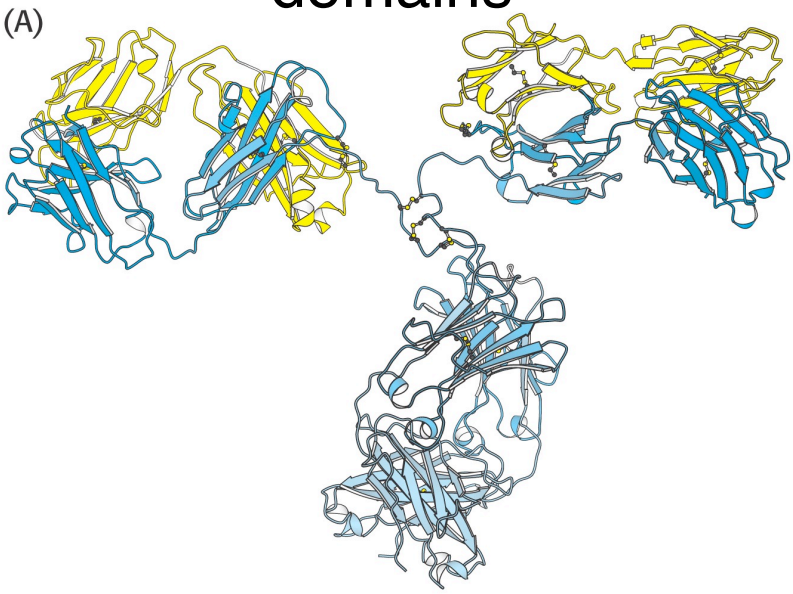
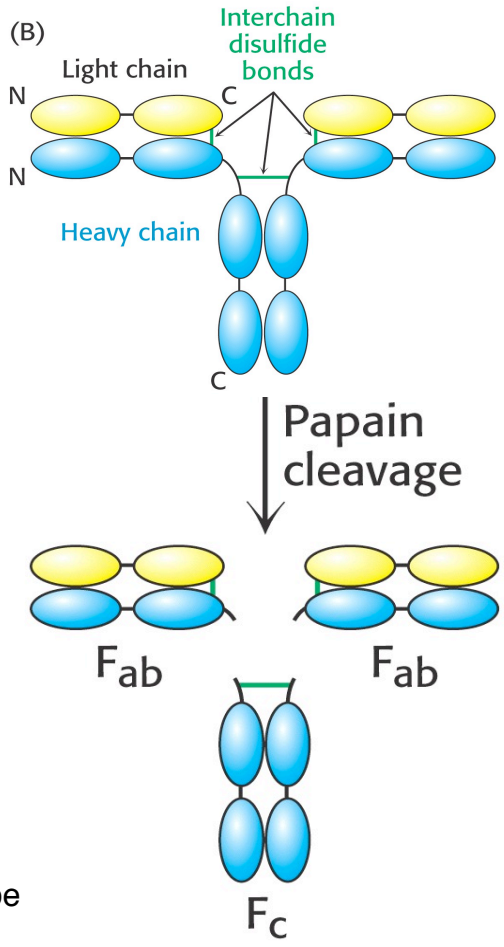
- The most amazing property of IgG is its ability to recognize such an incredibly diverse range of molecular species ranging from small molecules to large proteins.
- The molecular basis for this versatility is the ability of antibodies to tolerate a wide variety of amino acid changes in its antigen recognition site at the two tips of the ‘Y’.
- Each of the several billion antibodies circulating in your blood has a unique amino acid composition in this region of the antibody structure.
- But you only have ~3 billion base pairs of DNA…. How is it possible to encode such a large number of different gene products?

Immunoglobin G (IgG) has 4 polypeptide chains organized into 3 major domains

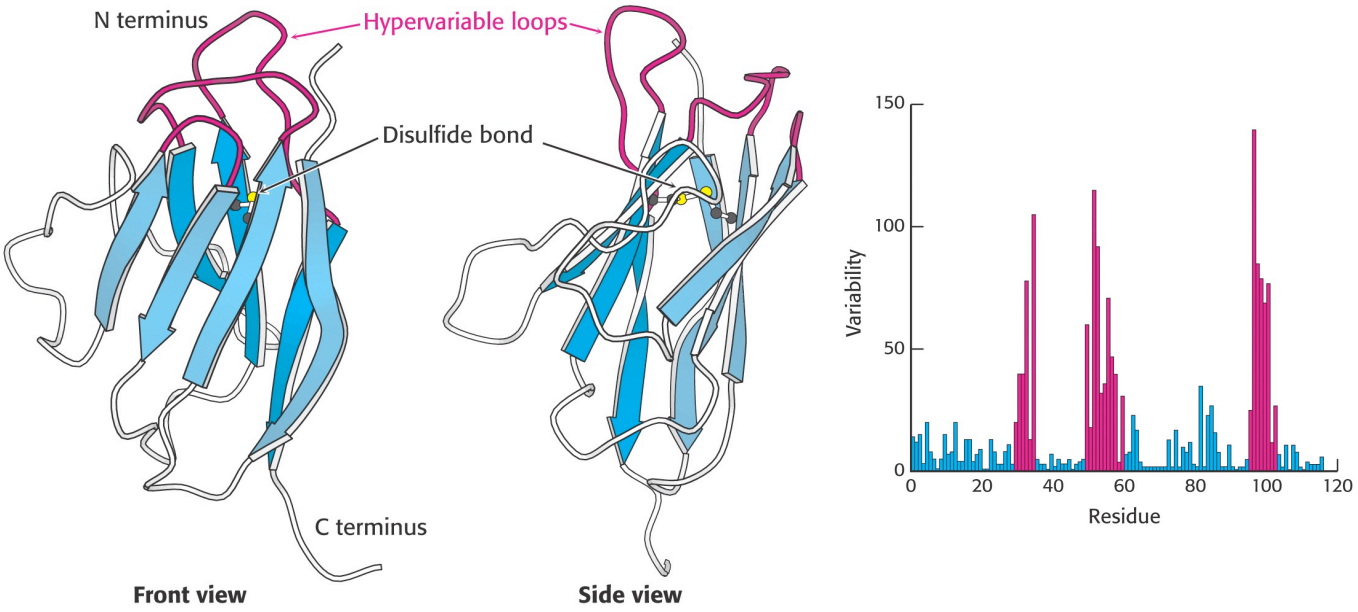




Why might flexible linkers between F_{ab} domains be important for antibody function?

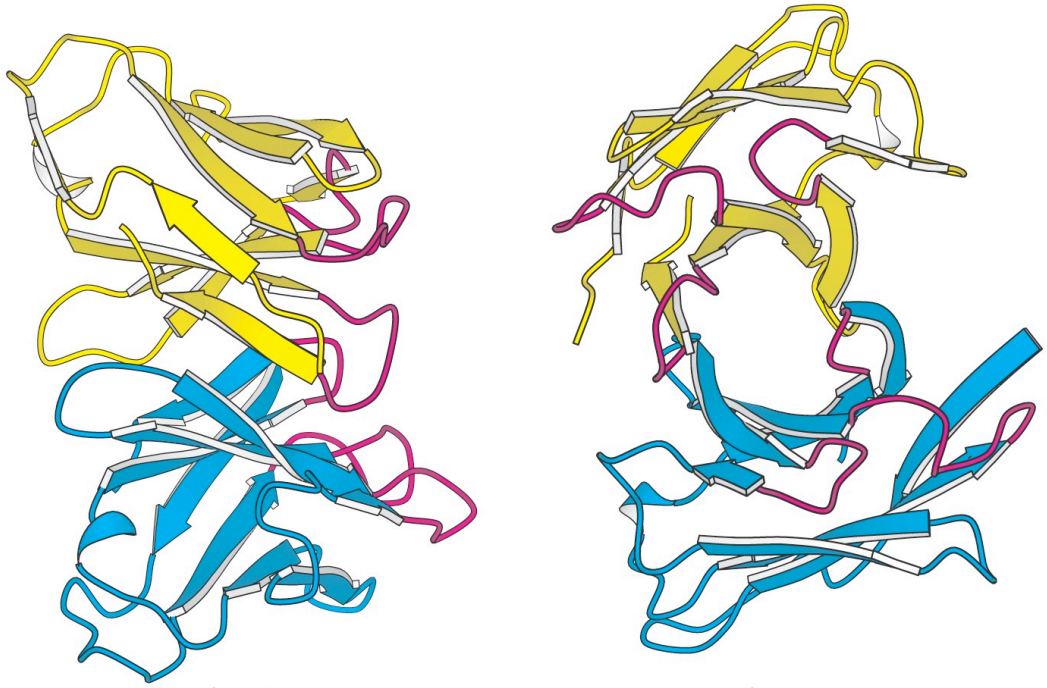
- In 1959, Rodney Porter showed that immunoglobulin G (IgG), the major antibody in serum, can be cleaved into three 50-kd fragments by the limited proteolytic action of papain (an enzyme that cleaves specific peptide bonds). Two of these fragments bind antigen. They are called Fab (F stands for fragment, ab for antigen binding). The other fragment, called Fc because it crystallizes readily, does not bind antigen, but it has other important biological activities.
- How do these fragments relate to the three-dimensional structure of whole IgG molecules? Immunoglobulin G consists of two kinds of polypeptide chains, a 25-kd light (L) chain and a 50-kd heavy (H) chain. The subunit composition is L2H2. Each L chain is linked to an H chain by a disulfide bond and non-covalent interactions, and the H chains are linked to each other by at least one disulfide bond plus non-covalent interactions.
- Each L chain comprises two homologous domains, termed immunoglobulin domains. Each H chain has four immunoglobulin domains. These domains have many sequence features in common and adopt a common structure, the immunoglobulin fold. The immunoglobulin fold is one of the most prevalent domains encoded by the human genome. More than 750 genes encode proteins with at least one immunoglobulin fold recognizable at the level of amino acid sequence.
- Overall, the molecule adopts a conformation that resembles the letter Y, in which the stem, corresponding to the Fc fragment obtained by cleavage with papain, consists of the two carboxyl-terminal immunoglobulin domains of each H chain and in which the two arms of the Y, corresponding to the two Fab fragments, are formed by the two amino-terminal domains of each H chain and the two amino-terminal domains of each L chain.
- The linkers between the stem and the two arms consist of relatively extended polypeptide regions within the H chains and are quite flexible.
- *Question: You say that the heavy chains and light chains of IgG are homodimers. But I think the tips of the two arms, the CDR, are different. How can they be deemed as homodimer?*
- *Answer: What I meant by this is that the IgG can be though of as a homodimer of a heterodimer (made of one heavy chain plus one light chain). The CDR regions at the end of each arm are identical for a given antibody.*
- *Question: For antibodies in an organism, is it right to say they are only different in Fv, the remaining parts are all the same*
- *Answer: As far as this course is concerned, this is correct.*

The VL and VH immunoglobulin domains each have 3 hypervariable loops



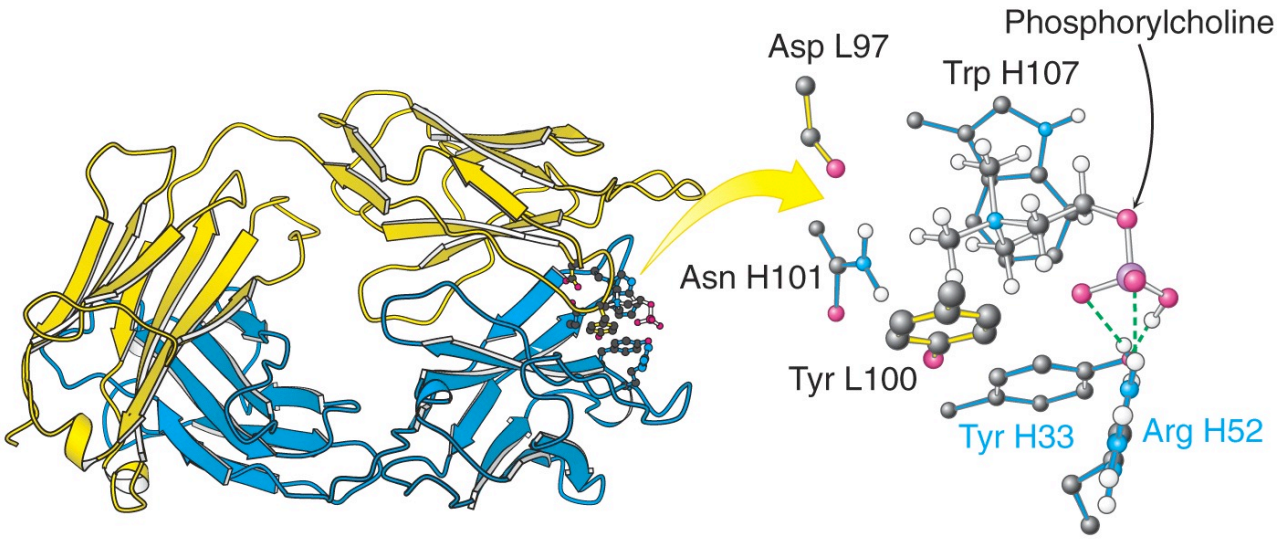
- The immunoglobulin fold consists of a pair of β-sheets, each built of antiparallel β-strands, that surround a central hydrophobic core. A disulfide bond bridges the two sheets.
- Two aspects of this structure are particularly important for its function.
- First, three loops present at one end of the structure form a potential binding surface. These loops contain the hypervariable sequences present in antibodies and in T-cell receptors. Variation of the amino acid sequences of these loops provides the major mechanism for the generation of the vastly diverse set of antibodies and T-cell receptors expressed by the immune system. These loops are referred to as hypervariable loops or complementarity determining regions (CDRs).
- Second, the amino terminus and the carboxyl terminus are at opposite ends of the structure, which allows structural domains to be strung together to form chains, as in the L and H chains of antibodies. If the termini werre on the same side of the domain, it is less likely that the domains could be strung together to make a chain since they would bump into each other.
- *Question: Is somatic recombination a random process where some genes between V, D and J are being deleted (in two steps). I was wondering is there any governing factor which decides which gene would get deleted?*
- *Answer: For the sake of this course it is safe to assume that it is a completely random process. To the best of my knowledge this is essentially correct.*

The complete ‘variable’ fragment is composed of two immunoglobulin domains, each with 3 hypervariable loops



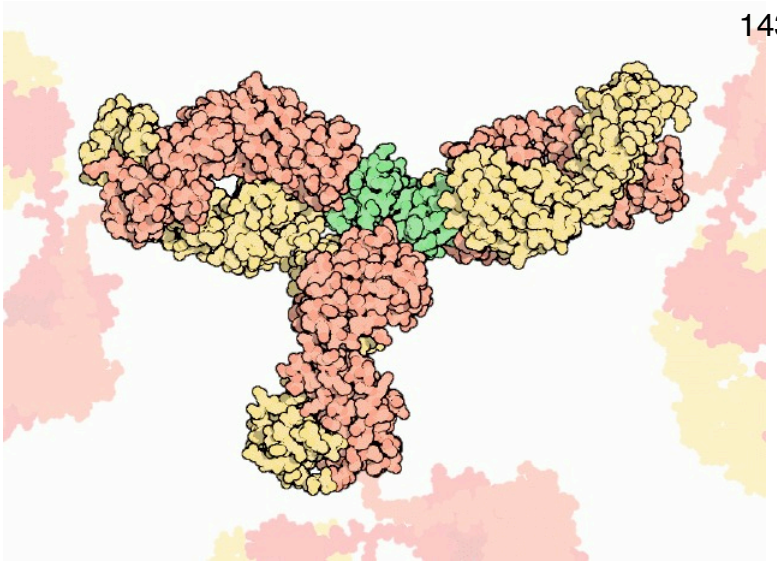
- The amino-terminal immunoglobin domains of the L and H chains (the variable domains, designated VL and VH) come together at the ends of the arms extending from the structure.
- The positions of the complementarity-determining regions are striking. These hypervariable sequences, present in three loops of each domain, come together so that all six loops form a single surface at the end of each arm. Because virtually any VL can pair with any VH, a very large number of different binding sites can be constructed by their combinatorial association.
- The results of x-ray crystallographic studies of many large and small antigens bound to Fab molecules have been sources of much insight into the structural basis of antibody specificity.
- The binding of antigens to antibodies is governed by the same principles that govern the binding of substrates to enzymes. The shape complementarity between the antigen and the binding site results in numerous contacts between amino acids at the binding surfaces of both molecules. Numerous hydrogen bonds, electrostatic interactions, and van der Waals interactions, reinforced by hydrophobic interactions, combine to give specific and strong binding.

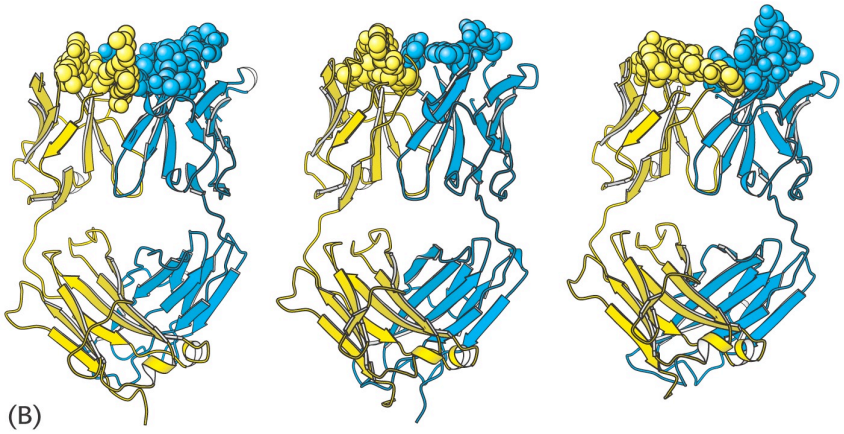
Antibodies can specifically bind small molecules in the antigen-binding region



- Small molecules often bind in a cleft of the antigen-binding region.
- A well-studied case of small-molecule binding is seen in an example of phosphorylcholine bound to Fab. Crystallographic analysis revealed phosphorylcholine bound to a cavity lined by residues from five CDRs — two from the L chain and three from the H chain.
- The positively charged trimethylammonium group of phosphorylcholine is buried inside the wedge-shaped cavity, where it interacts electrostatically with two negatively charged glutamate residues. The negatively charged phosphate group of phosphorylcholine binds to the positively charged guanidinium group of an arginine residue at the mouth of the crevice and to a nearby lysine residue. The phosphate group is also hydrogen bonded to the hydroxyl group of a tyrosine residue and to the guanidinium group of the arginine side chain. Numerous van der Waals interactions, such as those made by a tryptophan side chain, also stabilize this complex.
- The binding of phosphorylcholine does not significantly change the structure of the antibody, yet induced fit plays a role in the formation of many antibody-antigen complexes. A malleable binding site can accommodate many more kinds of ligands than can a rigid one. Thus, induced fit increases the repertoire of antibody specificities.

3 different Fab fragments recognize 3 different epitopes on lysozyme



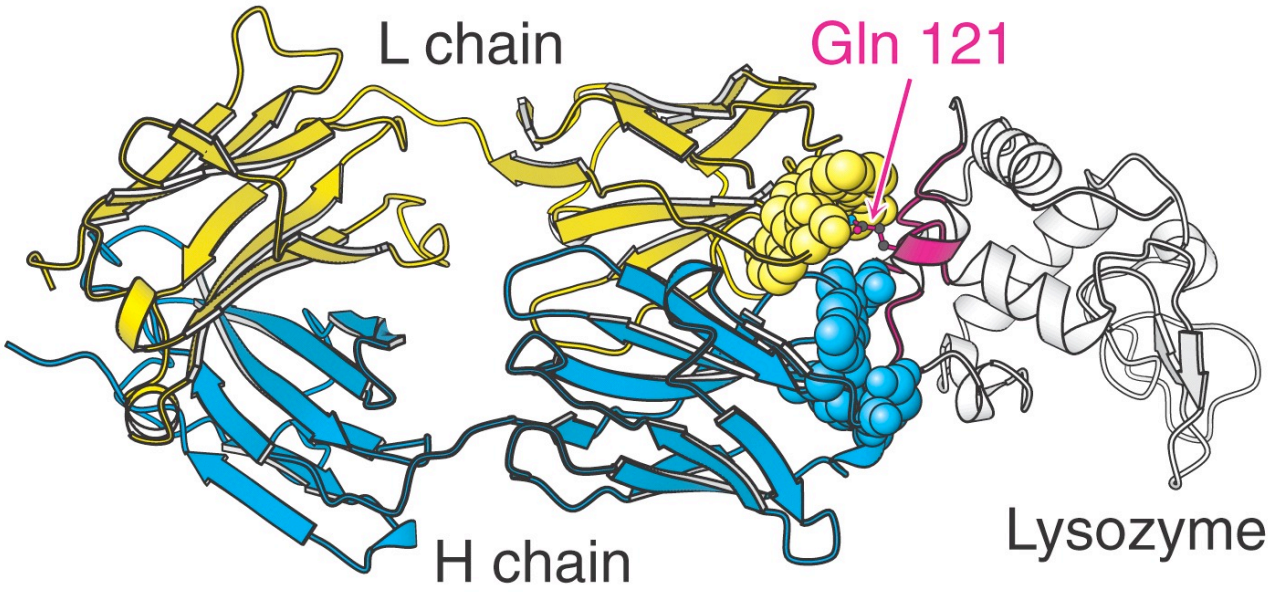


(B)

David S. Goodsell: The Molecule of the Month appearing at the PDB

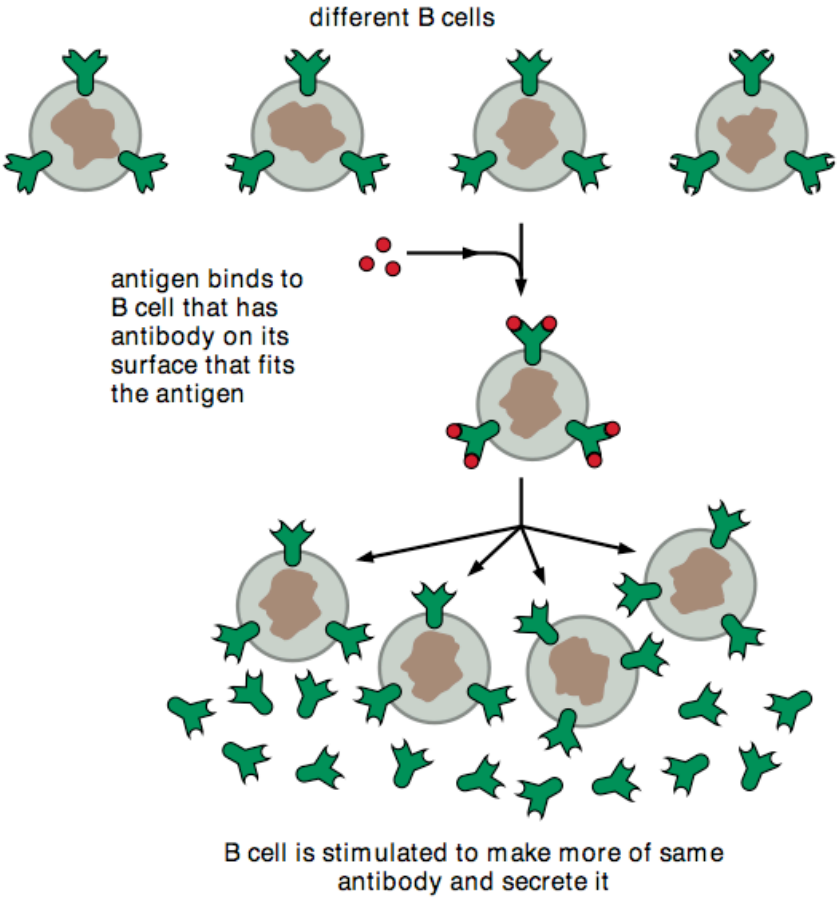
- A large collection of antibodies raised against hen egg-white lysozyme has been structurally characterized in great detail. Each different antibody binds to a distinct surface of lysozyme.
- The specific part of the protein to which the antibody binds is known as the epitope.
- The models on this slide show how one antigen has potentially many different epitopes.
- Note that a mixture of polyclonal antibodies would contain individual antibodies that bind to all possible epitopes of a given antigen. A monoclonal antibody would bind to only one specific epitope.

Detailed look at the epitope of one of the lysozyme:antibody complexes



- Let us examine the interactions present in one of these complexes in detail.
- This antibody binds two polypeptide segments that are widely separated in the primary structure, residues 18 to 27 and 116 to 129.
- This epitope is discontinuous in terms of primary structure, but is a continuous surface in the 3-dimensional structure.
- All six CDRs of the antibody make contact with this epitope. The region of contact is quite extensive. The contacting surfaces are rather flat. The only exception is the side chain of glutamine 121 of lysozyme, which penetrates deeply into the antibody binding site, where it forms a hydrogen bond with a main-chain carbonyl oxygen atom and is surrounded by three aromatic side chains.
- The formation of 12 hydrogen bonds and numerous van der Waals interactions contributes to the high affinity (Kd = 20 nM) of this antibody-antigen interaction.
- Examination of the Fab molecule without bound protein reveals that the structures of the VL and VH domains change little on binding, although they slide 1 Å apart to allow more intimate contact with lysozyme.

Where do all those antibodies come from?



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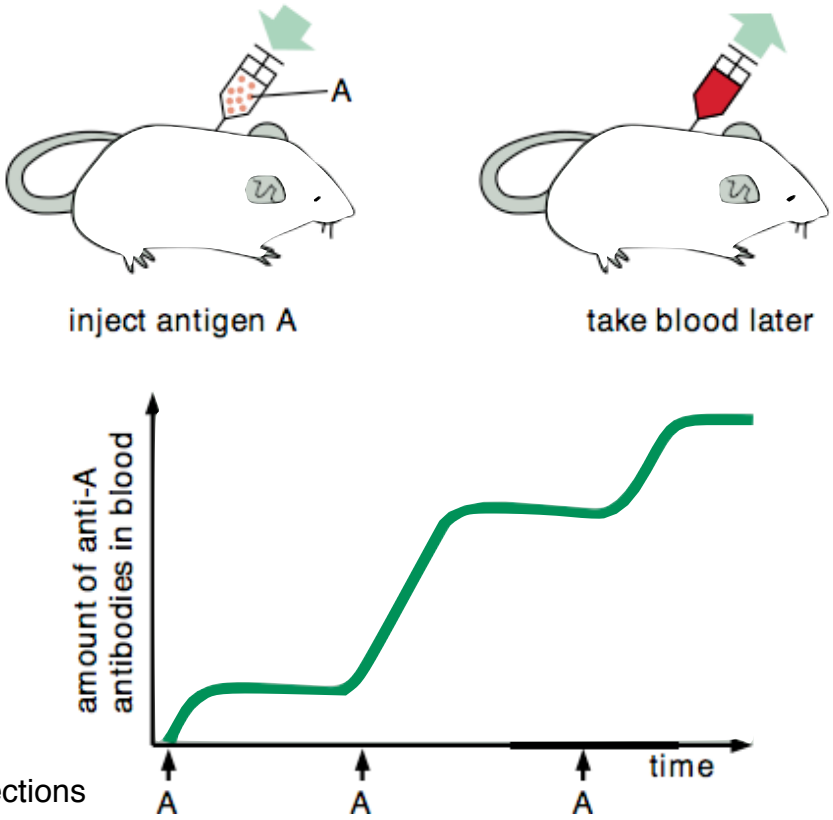
- Antibodies are made by a class of white blood cells, called B lymphocytes, or B cells.
- Each resting B cell carries a different membrane-bound antibody molecule on its surface that serves as a receptor for recognizing a specific antigen.
- When antigen binds to this receptor, the B cell is stimulated to divide and to secrete large amounts of the same antibody in a soluble form.
- Each B-cell has the ability to modify the sequence of the gene encoding its associated antibody molecule. This result of this process is that each B-cell expresses a unique antibody with a unique antigen binding site.
- In each B-cell, the antibody gene has been assembled from several variable ‘cassettes’ of DNA. The particular arrangement of multiple gene fragments known as V (variable), D (diversity), and J (joining) can give rise to millions of different gene products. Further random mutation of the gene introduces even greater diversity.
- This process is known as V(D)J recombination.

How do we make *polyclonal* antibodies for use in research?



A = new injections

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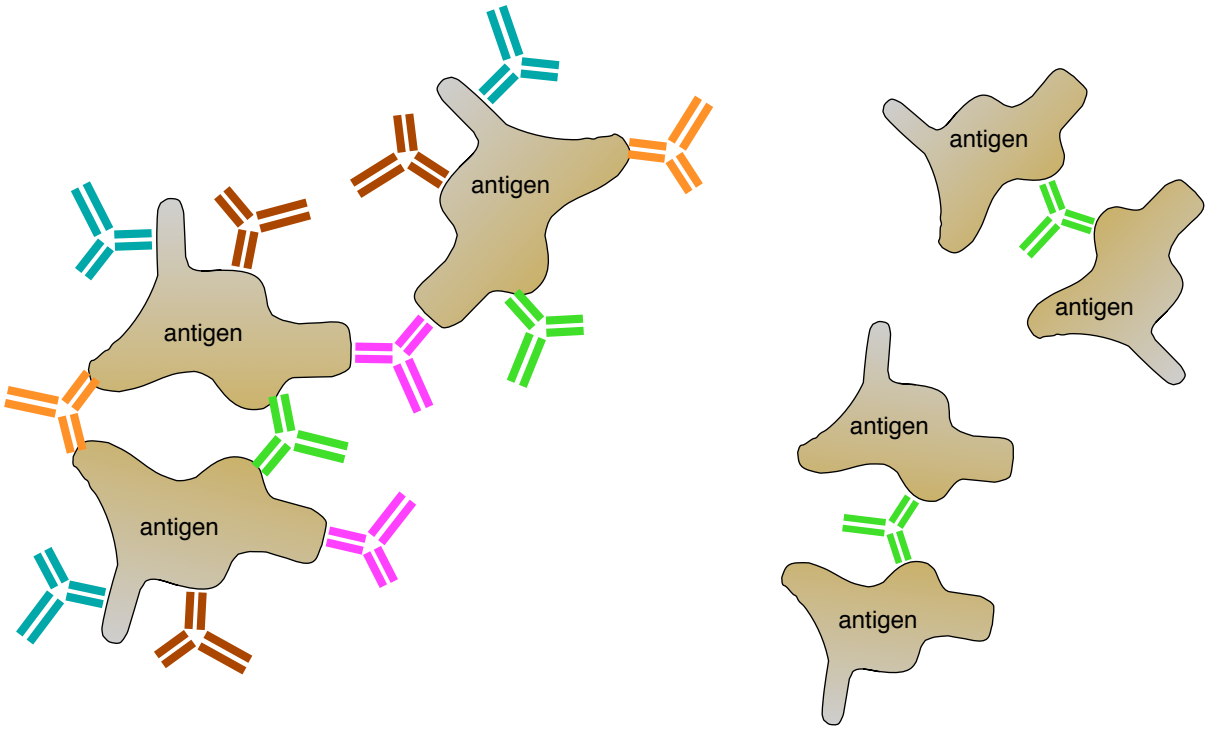
- Antibodies can be made in the laboratory by injecting an animal (usually a mouse, rabbit, sheep, or goat) with antigen (A).
- Repeated injections of the same antigen at intervals of several weeks stimulates specific B cells to secrete large amounts of anti-A antibodies into the bloodstream.
- Because many different B cells are stimulated by antigen A, the blood will contain a variety of anti-A antibodies, each of which binds A in a slightly different way.
- It is possible to prepare lots of antibodies (polyclonal) through affinity purification from the plasma of a previously immunized animal. The standard affinity purification would involve using a column on which either protein G or protein A (that is the name of an actual protein) is immobilized. These proteins are known to bind tightly to constant regions of IgG.
- A better affinity purification is to use an immobilized version of the same protein as was used to immunize the animal. For affinity purification the antigen would be immobilized on a resin. The antibody would be bound to the antigen on the resin and proteins that don't bind would be washed away. The antibody could then be eluted by a dramatic change in pH. For example 100 mM Glycine pH 2.5 for acid elution or 100 mM ethanolamine pH 11.5 for base elution.

Monoclonal vs. Polyclonal



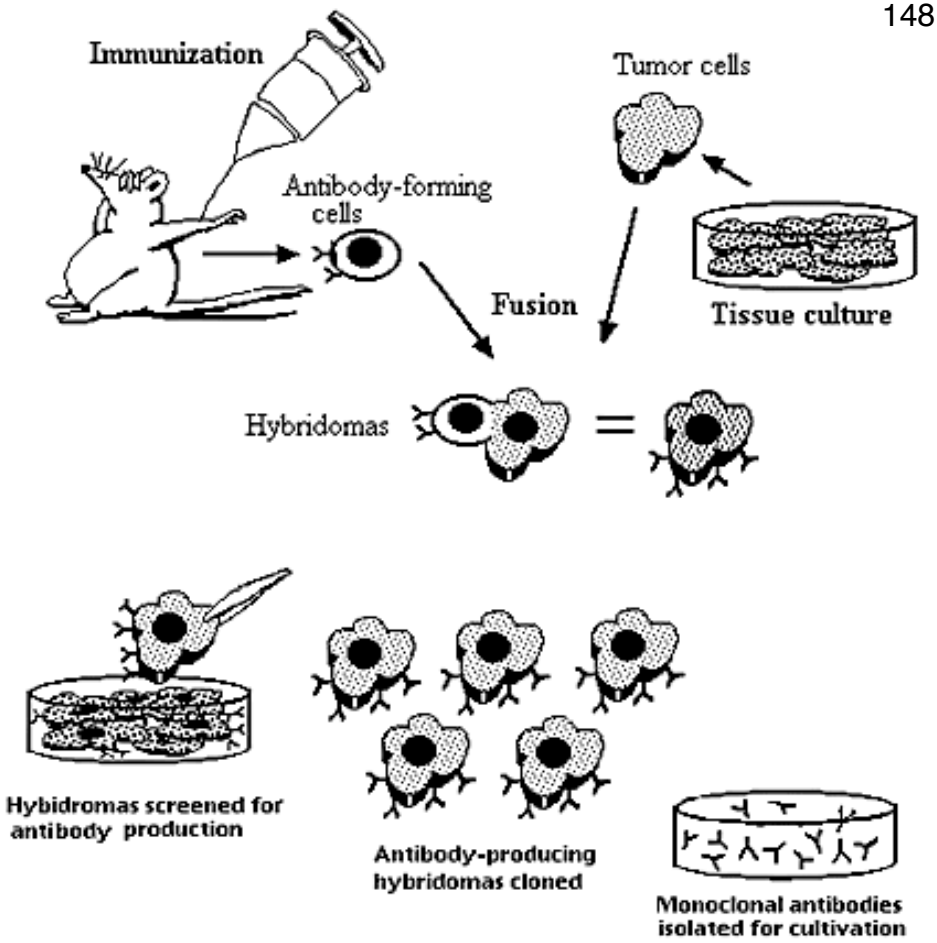
polyclonal antibodies

monoclonal antibody

More on monoclonals: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/M/Monoclonals.html

- Polyclonal antibodies are a mixture of many different antibodies with different affinities to different epitopes of the same antigen. It is technically incorrect to refer to a polyclonal antibody (that is, in the singular form) since ‘polyclonal’ implies many different molecular entities and so ‘antibodies’ is better than ‘antibody’
- A monoclonal antibody is a distinct antibody molecule that can only be prepared in a laboratory setting. An immune response always generates a mixture of antibodies. The trick is how do you isolate only one from this large population and then generate large quantities of it?

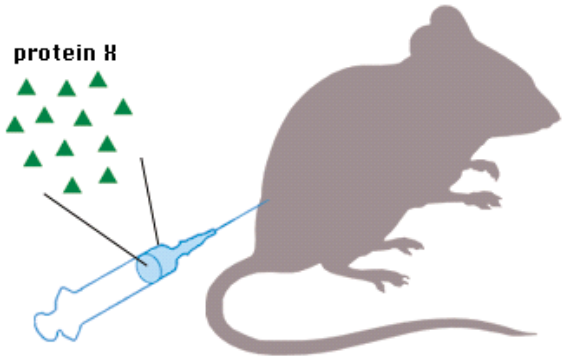
How do we make *monoclonal* antibodies?



Access Excellence

- This problem was solved by Köhler and Milstein in 1975. They received the Nobel prize in Medicine in 1984 for this work (http://www.nobel.se/medicine/laureates/1984/).
- A mouse is immunized by injection of an antigen A to stimulate the production of antibodies targeted against A.
- The antibody forming cells are isolated from the mouse's spleen.
- Monoclonal antibodies are produced by fusing single antibody-forming cells to tumor cells grown in culture. The resulting cell is called a hybridoma.
- Each hybridoma produces relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in culture, it is possible to produce a population of cells, each of which produces identical antibody molecules.
- These antibodies are called "monoclonal antibodies" because they are produced by the identical offspring of a single, cloned antibody producing cell. Once a monoclonal antibody is made, it can be used as a specific probe (i.e. in a western blot) to track down and purify the specific protein that induced its formation.
- Monoclonal antibodies are widely used as diagnostic and research reagents. Their introduction into human therapy has been much slower. In some in vivo applications, the antibody itself is sufficient. Once bound to its target, it triggers the normal effector mechanisms of the body. In other cases, the monoclonal antibody is coupled to another molecule, for example
  - a fluorescent molecule to aid in imaging the target
  - a strongly-radioactive atom, such as Iodine-131 to aid in killing the target.
- *Question: Why myeloma is used for producing monoclonal? Isn't it correct that, If the antigen is injected to a mouse, its body makes antibody and after getting its blood and separation of the antigen, we will have the antibody?*
- *Answer: The goal of monoclonal antibody production is the reproducible production of a single antibody (encoded by a single gene) with well-defined binding properties. One way of achieving this is to fuse the B-cells that make the antibodies with cancer cells that are immortal. The resulting fusion cells are immortal and each one makes one specific antibody. Once you identify a particular fusion cell that makes the 'best' antibody, you have a never ending supply of it. In contrast, if you purify the antibodies from the blood of a mouse you obtain many copies of many different antibodies (i.e., that bind to different epitopes with the antigen with a variety of affinities) all encoded by (slightly) different immunoglobulin genes.*

Why do we make hybridomas?



B-lymphocyte (cells that make antibodies but can't grow in a dish) **+** **Myeloma (cancer) cells** (cells that don't make antibodies but can grow in in a dish)

fusion and growth in HAT medium

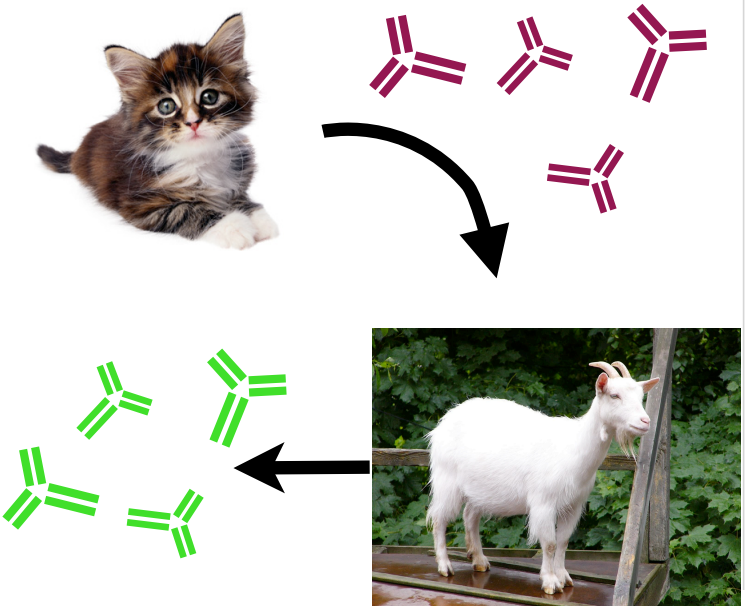
unfused B = B cell
B + B = BB cell
B + M = BM cell
M + M = MM cell
unfused M = M cell

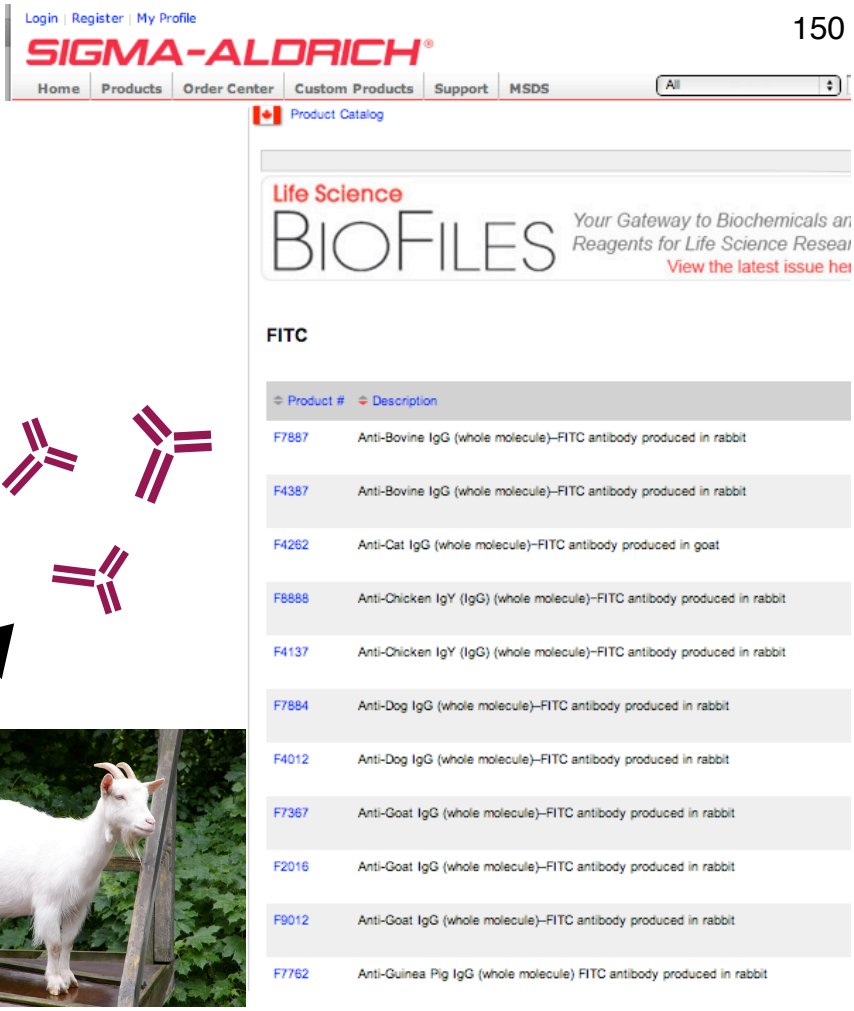
Only these cells will survive under these growth conditions, will produce antibodies, and will be immortalized!!

- A hybridoma is a cell that results from the fusion of a B-lymphocyte and a Myeloma (cancer) cell. The hybridoma cells have the properties of both of the cells that were originally fused to produce it.
- B-lymphocytes make the antibodies of interest to us, this is obviously the most important property that we want to preserve in the hybridoma. Unfortunately, B-lymphocytes can not grow indefinitely in a cell culture dish and so the cells will soon die. Another property of B-lymphocytes is that they have two different mechanisms for making GTP:
  - one process can be inhibited by a drug known as aminopterin. This process is also used for synthesis of TTP
  - another way is to use the enzyme Hypoxanthine-guanine phosphoribosyltransferase (HGPRT). This process is not inhibited by aminopterin.
- Myeloma (cancer cells) can grow indefinitely in a dish. They lack the HGPRT enzyme and will die in the presence of aminopterin.
- Following fusion, the hybridoma cells are grown in HAT medium (H - hypoxanthine, A - aminopterin, and T - thymidine)
- DNA synthesis requires synthesis of four nucleotides (ATP, GTP, TTP, CTP)
- B-lymphocytes that did not undergo fusion will die because they are not immortal
- Myeloma cells that did not undergo fusion will die because they can not make GTP
- Only the B-lymphocyte/Myeloma hybridomas will be able to survive since they are immortalized and can still make GTP using HGPRT
- The surviving cells are divided into multiwell plates such that each well has a single cell. After the cells have grown for a while, the growth medium of each well of the plate is tested for the antibody specificity of interest. Once it is found, that particular hybridoma can be indefinitely cultured and used to produce the monoclonal antibody indefinitely.
- *Question: If myeloma (cancer cells) will die in the presence of aminopterin., how does the fusion take place in HAT medium (H - hypoxanthine, A - aminopterin, and T - thymidine)?*
- *Answer: The fusion probably doesn't occur in exactly this media. It is likely that the fusion itself occurs in a different media that is then exchanged to the HAT medium*
- *Question: In the process of producing monoclonal antibodies, we should expose just one single B-cell of the mouse to tumor cell. How can we separate just one cell?*
- *Answer: Many thousands of hybridoma cells would be made and these would all survive in HAT medium. These cells are then dispensed into individual wells of multiwell plates and allowed to grow. The growth medium in each well is then tested for the presence of the antibody of interest.*

- http://people.rit.edu/gtfsbi/hytc/PDFfiles/HAT%20Medium.pdf
- http://web.virginia.edu/Heidi/chapter27/chp27.htm

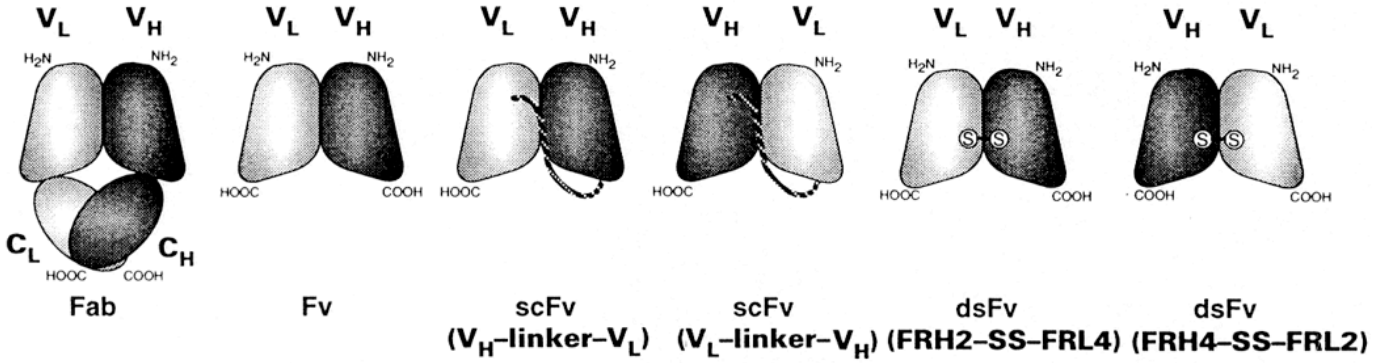
Antibodies from different species recognize each other as being foreign proteins





- As we've seen, the whole purpose of antibodies is to recognize foreign proteins that happen to find their way into the blood of an animal
- Although antibodies from all mammals are practically identical in terms of overall structure and function, there are still enough minor differences for them to be recognized as foreign when introduced into another species. For example, cat antibodies are recognized as foreign when introduced into a goat. And cow antibodies are recognized as foreign when introduced into a rabbit.
- So, immunizing an animal with antibodies from another species leads to the generation of 'anti-antibodies'. In the first example mentioned above, the antibodies that are made (and could be purified from the blood of the animal) would be called 'goat anti-cat IgG' or 'anti-cat IgG produced in goat', or something like that.
- These anti-antibodies are known as secondary antibodies and incredibly useful in bioanalytical chemistry applications.
- However, this does one interesting question: where do we get antibodies for human therapeutic applications? Many of the most sophisticated cancer therapies rely on treating patients with antibodies that target the cancer cells (through a variety of mechanisms). Where could these antibodies come from?

The Fab fragment can be further minimized by protein engineering to give just the variable (VL and VH) domains



http://www.unizh.ch/%7epluckth/publications/pdf/APpub0108.pdf

- A Fab fragment can be obtained by proteolysis of an antibody purified from hybridomas
- In contrast, all the Fv fragments must be generated by molecular biology techniques and expressed in bacteria.
- Note that the scFv fragments are single proteins, i.e. there is only one polypeptide chain.
- Fab, Fv, and dsFv are two polypeptide chains

How to make human antibodies for therapeutics?

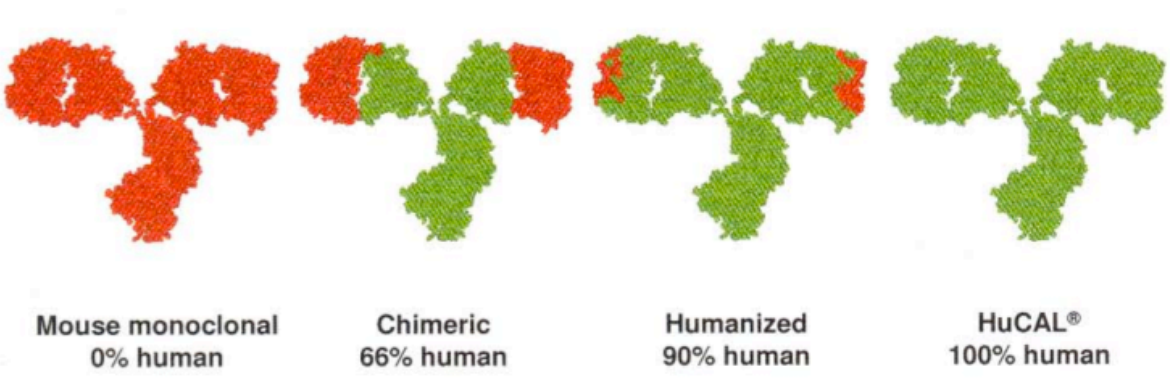


Fig. 2.1 Diagram showing the proportion of human (green) and murine (red) sequences in mouse, chimeric, humanized and human antibody structures, as exemplified by HuCAL.

Moroney, S., and Plückthun, A. (2005) in Modern Biopharmaceuticals: Modern Antibody Technology: The Impact on Drug Development (*Knäblein, J., ed*) Vol. 3, 1 Ed., pp. 1147-1186, 4 vols., Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

- Antibodies are now considered mainstream therapeutics and there are at least 21 different monoclonal antibodies now approved as human therapeutics. They are particularly useful for cancer therapy.
- The first antibodies tested for human therapeutic applications during the 1980s were of mouse origin. Immunogenicity was an obvious problem so researchers started looking for ways to create human antibodies. The most successful approach was to engineer a mouse antibody to look more “human”
- The first chimeric antibodies kept the mouse variable domains and the remainder was human. Several of these antibodies are now FDA approved. it seems that ~6-10% of patients will have an immune response to these antibodies.
- Another approach was to graft the CDRs from a mouse antibody onto a human antibody to produce something that is 90% human. This is a little bit trickier than it sounds because the framework influences the presentation of the loops.
- ‘Most of the growing number of antibodies entering clinical trials are completely human and are derived from phage-display technology or transgenic mice that express human immunoglobulin genes.’ (Paul J. Carter, Potent antibody therapeutics by design Nature Reviews Immunology 6, 343-357 (May 2006.

phage display

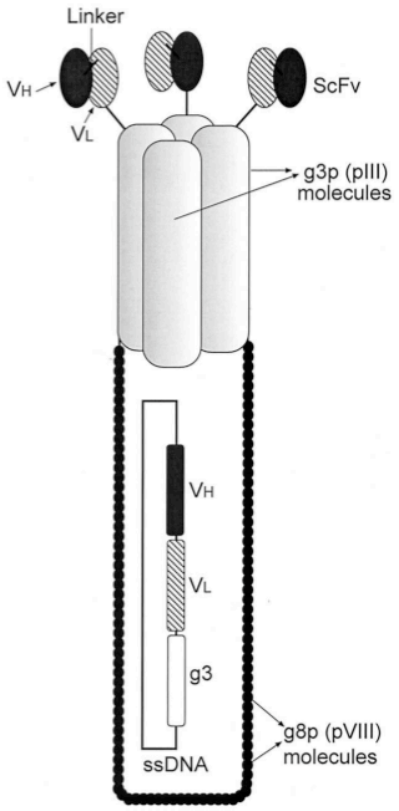


Fig. 1. Schematic diagram of a filamentous phage displaying single chain variable fragment (scFv) molecules. The phage consists of circular ssDNA surrounded by a coat protein. g8p (pVIII) is the major coat protein whereas g3p, at the tip of the phage, is one of the minor coat proteins. The genes encoding the variable domains of the scFv and a linker are fused to gene III (g3) in the genome of the filamentous phage. Consequently, the scFv is displayed as a fusion to g3p (pIII) protein at the tip of the phage. In reality, the scFv is not fused to all g3p protein molecules, and therefore the phage retains its ability to infect bacteria. Four g3p molecules are illustrated in the figure, three of which display scFv molecules.

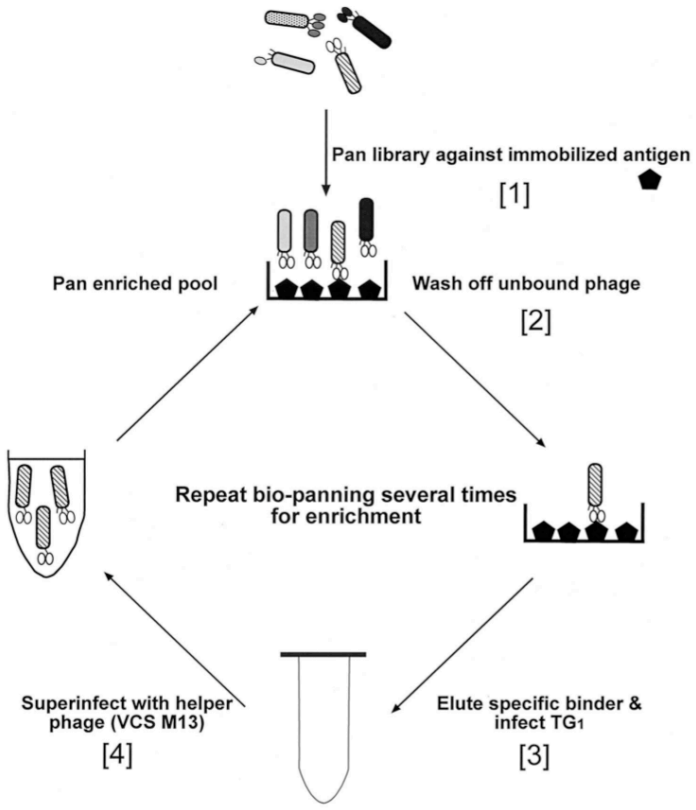
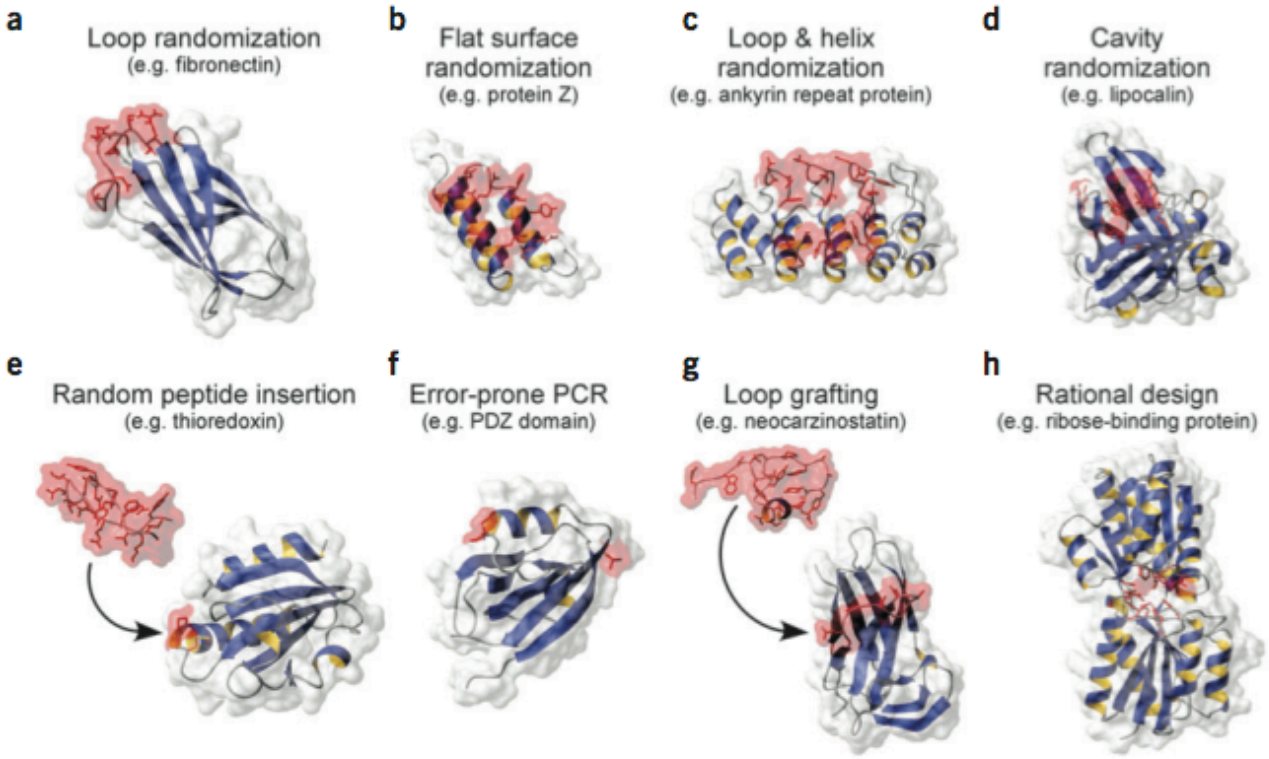


Fig. 4. Bio-panning of phage-display library. The library is screened in four steps: (1) binding of phages to the target antigen (in this case antigen immobilized on a solid support), (2) washing to remove unbound phage, (3) dissociation to recover antigen-specific phage, and (4) amplification of the antigen-specific phage by infection of *E. coli*. Caution must be exercised during the binding and washing steps to avoid low-ionic strength or other conditions that may favor adsorption of phage directly onto plastic or other matrix.

H.M.E. Azzazy, W.E. Highsmith Jr. / *Clinical Biochemistry* 35 (2002) 425– 445

- There are now a handful of in vitro methods for engineering antibodies with specific binding functions. These methods do not require that an animal be immunized. CDR diversity can be generated using synthetic portions of cDNA that are inserted into the gene encoding an ScFv.
- One of these techniques is known as phage display. A ‘phage’ or bacteriophage is a virus that infects bacteria. Accordingly, they are harmless to all animals including humans. Phage display uses a particular type of phage called a filamentous phage (typically M13). The reproduction of most viruses is more or less similar, so you can think about the life cycle of M13 as being similar to that of HIV. One important difference is that the circular genome of M13 does not need to incorporate into the genomic DNA of the bacteria host. It is kept separate inside the bacteria.
- The basic principle of phage display is that the gene library (encoding variants of a particular protein) is inserted into a plasmid such that it is fused in frame to a phage coat protein. This plasmid gene library is used to transform *E. coli*. The *E. coli* is infected with a phage that will reproduce inside the bacteria making more copies of itself. These new copies will do two important things:
    1. They will package the plasmid (actually called a phagemid) inside their filamentous structures
    2. They will incorporate some of the phage coat proteins that are fused to the variant protein (which is in turn encoded by the phagemid packaged inside the phage).
- A typical library size is ~10^8 different phage displaying different proteins. The phage that display a variant that happens to bind to the target can be enriched in a process called ‘bio-panning’.
- This processes can be repeated several times in order to dramatically enrich for binding domains with the desired specificity
- For those that are interested, see also the related techniques of ribosome display and mRNA display. (Binz, H. K., Amstutz, P., and Plückthun, A. (2005). Engineering novel binding proteins from nonimmunoglobulin domains. Nat. Biotechnol. 23, 1257-1268.)
- *Question: In the example of inserting the scfv genes into a phage, how do we go from dsDNA from the library to ssDNA in phage.*
- *Answer: The trick is that that there is a site in the plasmid called an f1 origin of replication. DNA polymerase can start from this site and just proceed around the plasmid in one direction. This is a so-called 'rolling circle' amplification where DNA polymerase just keeps going around and around making one new new strand of DNA. This single strand of DNA can be cut at appropriate sites and circularized to produce the circular ssDNA that gets packaged into the phage.*
- *Question: I am just wondering that is phage display used to generate human antibody? We can not inject antigen into human body, right? If so, how can we get the biased library that you mentioned for genes of scfv. And what do you mean by cycles for enrichment? Does that means we should repeat all the steps(from prepare gene library) or only repeat affinity chromatography?*
- *Answer: It's true that you couldn't normally make a biased library of human ScFvs. Though you could imagine a situation where an individual happens to have acquired HIV but fortunately developed a particularly powerful antibody against the virus. In principle, you could take some B cells from that person and then isolate the antibody by phage display. Generally speaking, the biased library would be for non-human antibodies.The cycles of enrichment mean taking the phage isolated after affinity purification, reinfecting E. coli, harvesting the phage, and then repeating the affinity purification. In principle, you could introduce a bit more randomization of the gene with each cycle to create a new library based on the best variants from the previous round. These cycles need to be done because the process is not perfect (i.e., non-specific binding of phage) and you are trying to discover a potentially very rare variant (1 in a million). By repeating these cycles you are enriching for the variant with the desired binding, so it will make up a large percentage of the isolated phage.*

non-immunoglobulin scaffolds enable different types of binding sites to be exploited



- But if we can do phage display, why use antibodies at all?
- We can use many different types of proteins as the scaffold for binding.
- Some will have advantages such as being small relative to antibodies (may be able to access sterically hindered epitopes). Some may fold in cytoplasm (no disulfide bonds)
- However, they will not be as well suited to therapeutic applications.
- Figure Legend. Binding-site engineering strategies used with different alternative scaffolds. (a–h) In combinatorial engineering approaches, sequences of a scaffold can be diversified at specified positions by means of defined randomized codons (e.g., in loops (a), flat surfaces (b), combinations of loops and helices (c), or cavities (d)), or a random peptide sequence is inserted into the scaffold (e), usually at a loop, or the scaffold sequence is randomized at undefined positions (f). Target-binding variants of the resulting libraries are subsequently isolated using selection or screening technologies. In rational engineering approaches, preexisting binding sequences (e.g. loops) have been grafted onto a novel scaffold (g), or binding sites have been engineered de novo into a suitable scaffold (h). The different engineering possibilities are illustrated by alternative binding molecules where the engineering in question has been applied: loop randomization (fibronectin), flat surface randomization (protein Z), loop and helix randomization (ankyrin repeat protein), cavity randomization (lipocalin), random peptide insertion (thioredoxin), error-prone PCR (PDZ domain), loop grafting (neocarzinostatin) and rational design (ribose-binding protein). Many other permutations of randomization strategies and scaffolds are conceivable; this figure illustrates each strategy with one published example.
- From: Binz, H. K., Amstutz, P., and Plückthun, A. (2005). Engineering novel binding proteins from nonimmunoglobulin domains. Nat. Biotechnol. 23, 1257-1268.

Summary of Antibodies:structure and function

- Antibodies are generated by B-cells that are in our blood. There are 100s of millions of individual cells each making a unique antibody with a unique antigen recognition site.
- When a B-cell encounters an antigen that it is able to bind with, it starts to make more copies of itself and more copies of its particular antibody.
- For research purposes, we can take advantage of this process and inject small animals with antigens of interest to us. The animal will make lots of antibody against the antigen.
- We can purify the antibody from the animals blood, isolate B-cells and create immortal cell lines that will continue to make antibody, or use molecular biology to clone the antibody gene from the B-cells.
- Antibodies are universal molecular recognition units that are central to therapeutic, diagnostic, and almost all biosensor applications.
- However, antibodies are not the only proteins that can be used for molecular recognition. There are various other ‘protein scaffolds’ that can be adapted for this purpose.
- We will learn about the many applications of antibodies in bioanalytical chemistry in future lectures.