D1 THE IMMUNE SYSTEM

Key Notes

Functions

The immune system has two main functions; to recognize invading pathogens and then to trigger pathways that will destroy them. The humoral immune system relies on B lymphocytes to produce soluble antibodies that will bind the foreign antigens. The cellular immune system uses killer T lymphocytes that recognize and destroy invading cells directly.

Primary and secondary immune responses

The primary immune response occurs on initial contact with a foreign antigen and results in production of immunoglobulin M (IgM) and then immunoglobulin G (IgG). If the same antigen is encountered again, immunological memory leads to a secondary immune response that produces a much more rapid and larger increase in specific IgG production.

Clonal selection theory

A large number of antibody-producing cells exist in an animal even before it encounters a foreign antigen, each cell producing only one specific antibody and displaying this on its cell surface. An antigen binds to cells that display antibodies with appropriate binding sites and causes proliferation of those cells to form clones of cells secreting the same antibody in high concentration.

Self-tolerance

Cells that produce antibody that reacts with normal body components are killed early in fetal life so that the adult animal normally is unable to make antibodies against self, a condition called self-tolerance.

Complement

Antibodies bound to an invading microorganism activate the complement system via the classical pathway. This consists of a cascade of proteolytic reactions leading to the formation of membrane attack complexes on the plasma membrane of the microorganism that cause its lysis. Polysaccharides on the surface of infecting microorganisms can also activate complement directly in the absence of antibody via the alternative pathway.

Related topics

Antibodies: an overview (D2)
Antibody synthesis (D3)
Transport of macromolecules (E4)

Functions

There are two vital functions of the immune system; recognition of an invading pathogen (disease-producing bacteria, fungi, protozoa and viruses) as being distinct from normal body components (and hence treated as foreign) and then the triggering of pathways that lead to destruction of the invader, such as activation of complement (see below) and phagocytic cells that engulf and digest the invading organism. The immune system may also be able to recognize and destroy abnormal cells that arise spontaneously in the body which would otherwise lead

to cancer, but the significance of this phenomenon in protecting against human tumors is still debatable. The key cells responsible for immunity in vertebrates are white blood cells called lymphocytes which arise from precursor (stem) cells in the bone marrow. There are two main parts of the immune system which interact to provide overall protection for the animal:

- the **humoral immune response** (*humor* is an ancient term meaning fluid) relies on the production of soluble proteins called antibodies (or immunoglobulins) by **B lymphocytes**, so called because the cells mature in the bone marrow. As a common shorthand nomenclature, B lymphocytes are often called simply **B cells**.
- The **cellular immune response** is mediated by **T lymphocytes**, so called because their maturation from stem cells occurs in the thymus. In cellular immunity it is the intact T lymphocytes themselves that are responsible for the recognition and killing of foreign invaders. These cells are the **cytotoxic T lymphocytes (CTL)**, also called **killer T cells**. Other T lymphocytes have another role; they provide essential help for B lymphocytes to produce antibodies and so are called **helper T cells**.

In both cellular and humoral immunity, recognition of the foreign invader depends upon the recognition of foreign macromolecules (proteins, carbohydrates, nucleic acids); these foreign components are called **antigens**.

Primary and secondary immune responses

The presence of a foreign antigen stimulates the production of a specific antibody in the bloodstream which will recognize and bind tightly to it. Antibody molecules fall into five main classes, responses as defined by their precise structure (see Topic D2). The antibody molecules first produced after antigen injection are in the immunoglobulin M class and so are called IgM molecules. However, about 10 days after antigen injection, the amount of IgM in the bloodstream (the **titer** of antibody) declines and there is a concurrent increase in another class of antibody called immunoglobulin G (IgG); see *Fig. 1*. This is called the **primary immune response**.

One of the most important features of the immune system is that, once an animal has encountered a particular pathogen, the system confers protection

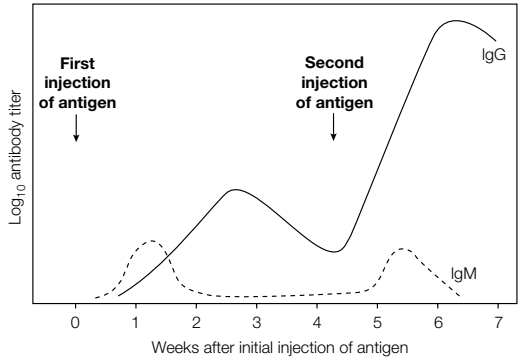

Fig 1. The primary and secondary immune responses to injections of antigen.

against future infection. This **immunological memory** means that if the same pathogen or antigen is encountered a second time, perhaps even decades after the previous occurrence, then the system reacts much faster and more dramatically to produce a large titer of specific IgG to counter the antigen. This is the **secondary immune response** (see *Fig. 1*) and is mediated by long-lived memory T cells and memory B cells.

Clonal selection theory

The **clonal selection theory** explains the operation of the humoral immune system as follows.

- Even before an animal meets a foreign antigen, each immature antibody-producing cell makes one (and only one) specific kind of antibody molecule and anchors this in the plasma membrane so that it is exposed on the cell surface. A large number of such antibody-producing cells exist in the animal which collectively express a very wide range of antibody specificities.
- If a foreign antigen is encountered, this will bind to those antibody-producing cells which are displaying antibody that has an appropriate binding site for that antigen. Each cell to which the antigen binds is stimulated to divide to form a population of identical cells called a **clone**. Since all the cells of the clone are genetically identical, they all produce antibody of the same specificity. The antibody now synthesized is no longer inserted into the plasma membrane but instead is secreted. Thus, when an antigen is encountered, it 'selects' certain antibody-producing cells for cell division (**clonal selection**) and antibody production simply on the basis of which cell surface antibodies bind that antigen. The specificity of the antibody produced is thus exquisitely tailored to the particular antigen(s) encountered.

Self-tolerance

In fetal life, if an immature antibody-producing cell displays cell surface antibody that binds a normal body component, then the cell dies. Thus, usually, in the adult animal, no cells exist that can make antibodies against the animal's own macromolecules. This inability to make antibodies against self is called **self-tolerance**. However, in some disease states (the so-called **autoimmune diseases**) the immune system loses its tolerance against self-antigens.

Complement

When the recognition function of the humoral immune system has been carried out by the production of specific antibodies and their binding to foreign antigens, destruction of the invading pathogen is the next step. One main defense pathway is the **complement system** which is activated by antibodies bound to the invading microorganism and eventually causes it to lyse by punching holes in its plasma membrane.

The complement system consists of about 20 interacting soluble proteins that circulate in the blood and extracellular fluid. Immunoglobulin molecules bound to the surface of the microorganisms activate **C1**, the first component of the complement pathway. The activation occurs through the Fc portion (see Topic D2) of the bound antibody. Only bound antibody can activate complement, soluble antibody not bound to an antigen has no such effect.

The early components of the complement pathway, including C1, are proteases that activate their substrate by limited cleavage. Activated C1 now activates several molecules of the next component by proteolysis, each of which activates several molecules of the next component by proteolysis, and so on. Therefore, the early steps in complement activation consist of a **proteolytic**

cascade in which more and more molecules are activated at each step. Component C3 is the key component whose cleavage leads to the assembly of **membrane attack complexes** on the plasma membrane of the microorganisms, which create holes in the plasma membrane that lead to cell death. Various white blood cells also become activated during this process and phagocytose (see Topic E4) the pathogen.

This pathway of complement activation, which starts when antibody has bound to antigen on the microbe surface, is called the **classical pathway**. An **alternative pathway** of activation also exists which is activated directly by polysaccharides in the cell wall of microorganisms even in the absence of antibody. The alternative pathway therefore defends the body against attack in the early stages before an immune response can occur and also augments the effects of the classical pathway of complement activation when the immune response has occurred.

D2 ANTIBODIES: AN OVERVIEW

Key Notes

Light and heavy chains

Each IgG antibody molecule consists of four polypeptide chains (two identical light chains and two identical heavy chains joined by disulfide bonds) and has two antigen-binding sites (i.e. is bivalent).

Variable and constant regions

Each light chain and each heavy chain consists of a variable region and a constant region. Variability in the variable regions is largely confined to three hypervariable regions; the remaining parts of the variable regions are far less variable and are called the framework regions.

Antibody domains

Each light chain folds into two domains, one for the variable region and one for the constant region. Each IgG heavy chain folds into four domains, one for the variable region and three in the constant region.

Fab and Fc fragments

Papain digests IgG into two Fab fragments (each of which has an antigen-binding site, i.e. is univalent) and one Fc fragment (that carries effector sites for complement activation and phagocytosis). Pepsin digests IgG to release an $F(ab')_2$ fragment that has two antigen-binding sites.

Five classes of immunoglobulins

Human immunoglobulins exist as IgA, IgD, IgE, IgG and IgM classes which contain α, δ, ε, γ and μ heavy chains, respectively. IgM is a pentamer that binds to invading microorganisms and activates complement killing of the cells and phagocytosis. IgG is the main antibody found in the blood after antigen stimulation and also has the ability to cross the placenta. IgA mainly functions in body secretions. IgE provides immunity against some parasites but is also responsible for the clinical symptoms of allergic reactions. The exact role of IgD is unknown. All antibody molecules contain either kappa (κ) or lambda (λ) light chains.

Polyclonal antibodies

A preparation of antibody molecules that arises from several different clones of cells is called a polyclonal antibody. It is a mixture of antibody molecules that bind to different parts of the antigen and with different binding affinities.

Monoclonal antibodies

Antibody produced by a single clone of cells is a monoclonal antibody; all the antibody molecules are identical and bind to the same antigenic site with identical binding affinities. Monoclonal antibodies can be generated in large amounts by creating a cell fusion (called a hybridoma) between an antibody-producing cell and a myeloma cell.

Related topics

The immune system (D1)
Antibody synthesis (D3)
Antibodies as tools (D4)

Light and heavy chains

Each molecule of immunoglobulin G (IgG) is Y-shaped and consists of four polypeptide chains joined together by disulfide bonds; two identical copies of

light (L) chains about 220 amino acids long and two identical copies of **heavy (H) chains** about 440 amino acids long (*Fig. 1a*). The N-terminal ends of one heavy chain and its neighboring light chain cooperate to form an antigen binding site, so that the IgG molecule has two binding sites for antigen, that is, it is **bivalent**. Because of this, a single antibody molecule can bind two antigen molecules and so cross-link and precipitate antigens out of solution.

Variable and constant regions

Comparison of the amino acid sequences of many immunoglobulin polypeptides has shown that each light chain has a **variable region** at its N-terminal end and an invariant or **constant region** at its C terminus (*Fig. 1b*). Similarly each heavy chain has an N-terminal variable region and a C-terminal constant region. Since it is the N-terminal parts of the light and heavy chains that form the antigen-binding site, the variability in amino acid sequence of these regions explains how different sites with different specificities for antigen binding can be formed. In fact, the variability in the variable regions of both light and heavy chains is mainly localized to three **hypervariable regions** in each chain (*Fig. 1b*). In the three-dimensional structure of the immunoglobulin molecule, the hypervariable parts of the light and heavy chains are looped together to form the antigen-binding site. The remaining parts of each variable region stay reasonably constant in sequence, usually do not contact the antigen directly and are called **framework regions**.

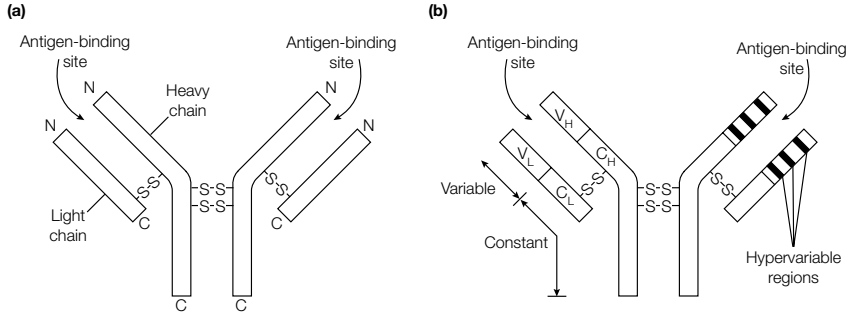

Fig. 1. Structure of an antibody molecule. (a) Each antibody molecule consists of two identical light chains and two identical heavy chains. The molecule has two antigen-binding sites, each formed by a light chain and a heavy chain. (b) The N-terminal regions of the light and heavy chains are variable in amino acid sequence from antibody to antibody (variable regions; V regions) whilst the C-terminal regions are relatively constant in sequence (constant regions; C regions). The generic terms for these regions in the light chain are V_L and C_L and for the heavy chains are V_H and C_H.

Antibody domains

Each light chain consists of two repeating segments of about 110 amino acids that fold into two compact three-dimensional **domains**, one representing the variable region of the light chain and the other domain representing the constant region. Each heavy chain is also made up of repeating units about 110 amino acids long. Since each IgG heavy chain is about 440 amino acids long, it forms four domains, one domain for the variable region and three domains in the constant region. The similarity of amino acid sequence between the various domains suggests that they arose in evolution by gene duplication.

Fab and Fc fragments

Papain, a protease, cuts the IgG molecule to release the two arms of the Y-shaped molecule, each of which has one antigen-binding site and is called an **Fab fragment** (Fragment antigen binding) (*Fig. 2*). Because Fab fragments have only one antigen-binding site (i.e. are **univalent**), they cannot cross-link antigens. The released stem of the Y-shaped molecule (consisting of the identical C-terminal parts of the two H chains) is named the **Fc fragment** (so called because it readily crystallizes). The Fc fragment carries the effector sites that trigger the destruction of the antigen, for example triggering of the complement system (see Topic D1) and inducing phagocytosis of pathogens by other white blood cells. In contrast to papain, pepsin (another protease) cuts the IgG molecule to release the two arms of IgG still linked together and hence this fragment has two antigen-binding sites (i.e. is bivalent) and can still cross-link antigens. This is called the **F(ab′)$_2$ fragment** (*Fig. 2*).

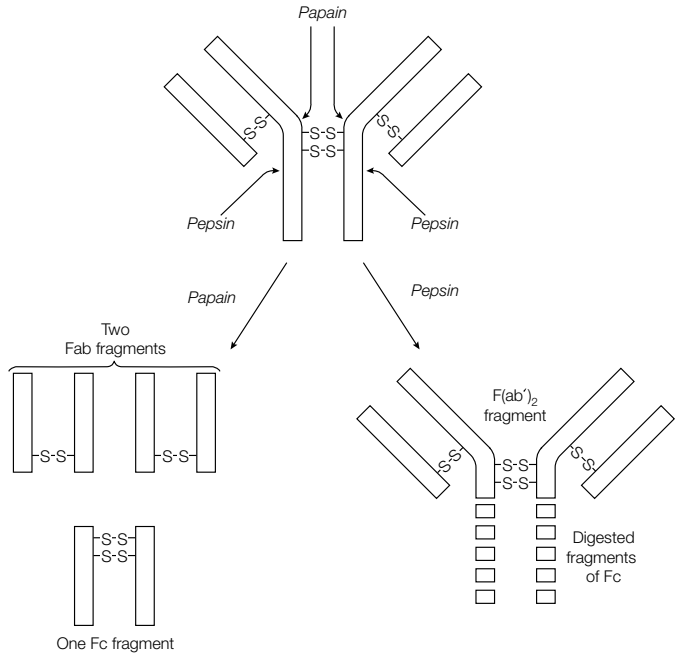

Fig. 2. Papain digestion of an antibody molecule yields two univalent Fab fragments and an Fc fragment whereas pepsin digestion yields a bivalent F(ab′)$_2$ fragment.

Five classes of immunoglobulins

Humans have five different classes of antibody molecule which differ both in structure and in function. These are called immunoglobulin A (IgA), IgD, IgE, IgG and IgM and each has its own type of heavy chain; α, δ, ϵ, γ and μ, respectively. Thus IgA molecules have two identical α heavy chains, IgD molecules have two identical δ heavy chains, etc. The human IgG class of antibodies is further divided into four IgG subclasses; IgG_1, IgG_2, IgG_3 and IgG_4, having γ_1, γ_2, γ_3 and γ_4 heavy chains respectively.

The different heavy chains confer different properties and functions on each of the immunoglobulin classes:

- **IgM** has μ heavy chains and exists as a pentamer in combination with another polypeptide called the J chain, which is responsible for initiating the polymerization to form the pentameric structure. With its large number of antigen-binding sites, each IgM molecule binds very tightly to any pathogen that has multiple copies of the same antigen on its surface. The binding induces the Fc region to activate the complement pathway which eventually causes the death of the pathogen. IgM also activates macrophages to phagocytose pathogens. Not surprisingly given these functions, IgM is the first antibody produced when an animal responds to a new antigen.
- **IgG** is the main immunoglobulin in the bloodstream late in the primary immune response and particularly during the secondary immune response (see Topic D1). Like IgM, it can activate complement and trigger macrophages, but is the only antibody that can pass through the placenta and so provide immunological protection for the fetus. It is also secreted into the mother's milk and is taken up from the gut of the newborn animal into the bloodstream, thus providing continuing protection after birth.
- **IgA** is the main class of antibody in secretions such as tears, saliva, and in secretions of the lungs and the intestine. It is the first line of immunological defense against infection at these sites.
- **IgE** occurs in tissues where, having bound the antigen, it stimulates mast cells to release a range of factors. Some of these in turn activate white blood cells (called eosinophils) to kill various types of parasite. However, the mast cells can also release biologically active amines, including histamine, which cause dilation and increased permeability of blood vessels and lead to the symptoms seen in allergic reactions such as hay fever and asthma.
- **IgD** is found on the surface of mature B lymphocytes and in traces in various body fluids, but its exact function remains unclear.

Two different forms of light chains also exist. Antibody molecules in any of the antibody classes or subclasses can have either two κ light chains or two λ light chains. Unlike the different heavy chains described above, no difference in biological function between κ and λ light chains is known.

Polyclonal antibodies

If an antigen is injected into an animal, a number of antibody-producing cells will bind that antigen (see Topic D1), albeit with varying degrees of affinity, and so the antibody which appears in the bloodstream will have arisen from several clones of cells, that is it will be a **polyclonal antibody**. Different antibody molecules in a preparation of polyclonal antibody will bind to different parts of the macromolecular antigen and will do so with different binding affinities. The binding region recognized by any one antibody molecule is called an **epitope**. Most antibodies recognize particular surface structures in a protein rather than specific amino acid sequences (i.e. the epitopes are defined by the conformation of the protein antigen). A preparation of polyclonal antibodies will bind to many epitopes on the protein antigen.

Monoclonal antibodies

If a single clone of antibody-producing cells (see above and Topic D3) could be isolated, then all of the antibody produced from that clone would be identical; all antibody molecules in such a **monoclonal antibody** preparation would bind to the same antigen epitope.

The problem is that if an individual antibody-producing cell is isolated and grown in culture, its descendants have a limited life-span that severely limits their use for the routine preparation of monoclonal antibodies. In 1975, Milstein and Köhler discovered how monoclonal antibodies of almost any desired antigen specificity can be produced indefinitely and in large quantities. Their method was to fuse a B lymphocyte producing antibody of the desired specificity with a cell derived from a cancerous lymphocyte tumor, called a myeloma cell, which is immortal. The cell fusion is called a **hybridoma**, which is both immortal and secretes the same specific antibody originally encoded by the B lymphocyte.

Monoclonal antibodies produced using this technology are now common tools in research because of their very high specificity. For example, they can be used to locate particular molecules within cells or particular amino acid sequences within proteins. If they are first bound to an insoluble matrix, they are also extremely useful for binding to and hence purifying the particular molecule from crude cell extracts or fractions (see Topic D4). They are also increasingly of use in medicine, both for diagnosis and as therapeutic tools, for example to inactivate bacterial toxins and to treat certain forms of cancer.

D3 ANTIBODY SYNTHESIS

Key Notes

Somatic recombination

No complete antibody gene exists in germ-line cells. The genes for light chains and heavy chains assemble by somatic recombination during B-lymphocyte maturation.

Recombination of light chain genes

In the germ-line, each light chain gene exists as multiple V and J gene segments upstream of a single C gene segment. During B-lymphocyte differentiation, one V gene segment joins with one J gene segment (VJ joining) to assemble the complete light chain gene, usually by deletion of intervening DNA.

Recombination of heavy chain genes

Heavy chains are encoded by multiple V, J and D gene segments which lie upstream of a single copy of C gene segments for each of the constant regions of μ, δ, γ, ε and α chains. During B-lymphocyte differentiation, a D gene segment joins a J segment (DJ joining) and then the recombined DJ joins a V gene segment (VDJ joining).

Class switching

A B lymphocyte can change the class of antibody being expressed by moving a new C gene segment into position after the recombined VDJ segment, deleting the intervening DNA. The new heavy chain has a different constant region but retains the same antigen-binding specificity of the previous heavy chain.

Related topics The immune system (D1) Antibodies: an overview (D2)

Somatic recombination

In most animals, it is possible to distinguish **germ-line** cells from **somatic** cells. The germ-line cells are those that give rise to the male and female **gametes** (sperm and ova, respectively) whilst the somatic cells form the rest of the body structures of the individual animal. The importance of the germ-line cells is that it is these that ultimately give rise to the next generation.

The human genome is thought to contain fewer than 10^5 genes, yet a human can make at least 10^{15} different types of antibody in terms of antigen-binding specificity. Clearly the number of genes is far too small to account for most of this antibody diversity. Thus a **germ-line hypothesis**, whereby all antibodies are encoded by genes in germ-line cells, must be incorrect. In fact, the genes exist in separate coding sections and are assembled during B-lymphocyte maturation by a process called **somatic recombination.** This process of assembly takes place in every B lymphocyte. By assembling different fragments of DNA, completely new immunoglobulin genes can be created and hence this gives an enormous potential reservoir of antibody diversity.

Recombination of light chain genes

The variable (V) region of a κ **light chain** is encoded by a separate DNA sequence from that encoding the constant (C) region. These two segments lie on the same chromosome but they are sited some way apart. During maturation of

the B lymphocyte, the V and C region DNAs are moved next to each other and are joined together to create a functioning light chain gene (*Fig. 1*). This somatic recombination usually occurs by deletion of the DNA between the germ-line V and C regions but can also occur in some cases by an inversion mechanism.

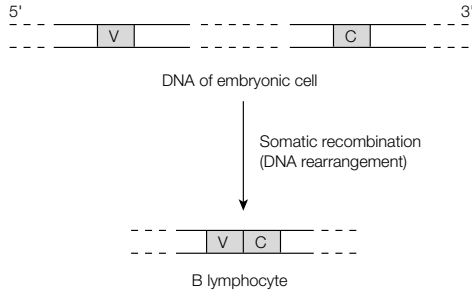

Fig. 1. In the germ-line (embryo) DNA, sequences coding for the variable (V) region lie distant from those encoding the constant (C) region. During the differentiation of B lymphocytes, these two sequences are brought together to form an active antibody gene by deletion of the intervening DNA (somatic recombination).

In fact, the situation is rather more complex than this simple model implies. The germ-line **V gene segment** (shown in *Fig. 1*) encodes only about the first 97 amino acids of the variable region of the light chain polypeptide. The remaining few amino acids of the light chain variable region (residues 98–110) are encoded by a piece of DNA called the **J gene segment** (*Fig. 2*) This J segment (for 'joining') must not be confused with the J chain in IgM pentamers (see Topic D2). In the germ-line, the J gene segment lies just upstream of the **C gene segment** and separated from it only by an intron (*Fig. 3*). Furthermore, there are multiple V and J gene segments; in humans about 40 V and five J regions exist (*Fig. 3*).

During B-lymphocyte differentiation, one of the 40 or so V regions becomes joined precisely to one of the J gene segments to create a light chain gene. This somatic recombination process is called **VJ joining**. In *Fig. 3*, V_3 has been chosen for recombination and has joined to J_2. Transcription now starts just upstream of the recombined V segment (just upstream of V_3 in *Fig. 3*) and continues until the end of the C segment. The other J segment sequences are also transcribed, but these sequences are lost during subsequent RNA splicing that removes the intron upstream of the C segment (*Fig. 3*). Thus the final mRNA contains only V_3J_2C sequences and encodes a corresponding light chain polypeptide. Overall, about 200 different human κ light chains can be made by joining any one of the 40 V segments to any one of the J gene segments.

The λ **light chain genes** also arise by somatic recombination during maturation of the B lymphocyte, but there are fewer V and J gene segments than for κ chain genes. Most antibody molecules have κ light chains and not λ light chains.

Recombination of heavy chain genes

Heavy chains are synthesized in an analogous manner but are encoded by four gene segments, V, J, D and C (*Fig. 4*). In humans, there are 51 V_H segments, 27 D_H segments (D for diversity, H for heavy) and six J_H segments. Thus, whereas the

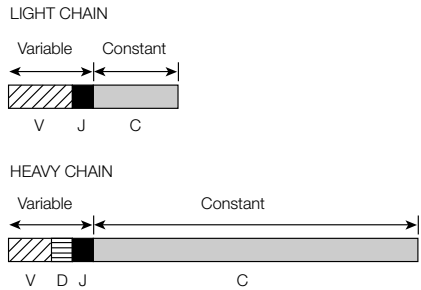

Fig. 2. The light chain variable region is encoded by two separate gene segments, V and J. The heavy chain variable region is encoded by three gene segments, V, D and J.

variable region of a light chain polypeptide is encoded by V and J segments, the variable region of a heavy chain is encoded by V, D and J segments (*Fig. 2*). In humans, joining any of the V, D and J segments generates $51 \times 27 \times 6 = 8262$ possible heavy chain variable regions. In the heavy chain gene system, there are

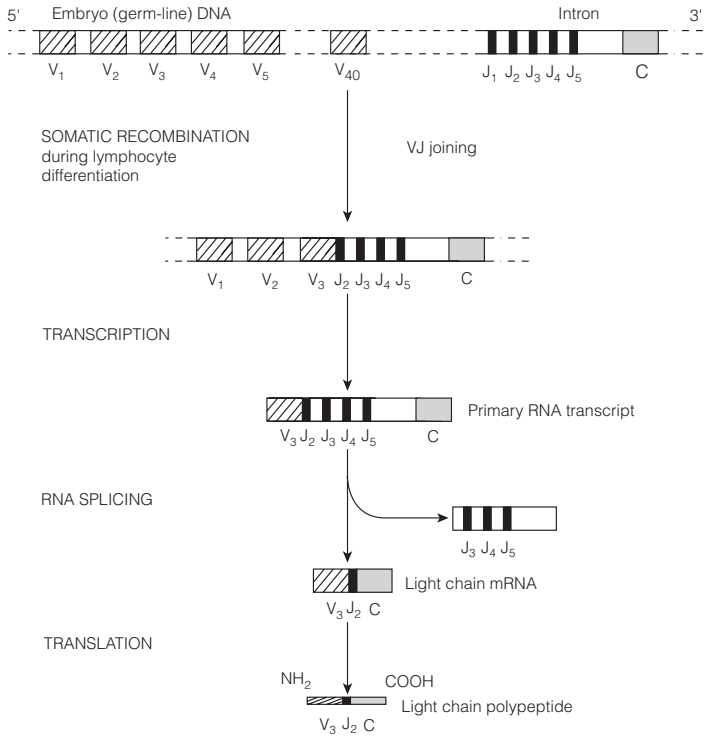

Fig. 3. Somatic recombination to create a human κ light chain gene and expression of that gene to produce κ light chain polypeptides.

also several C segments, one for each class of heavy chain; C_μ, C_δ, the various C_γ subclasses, C_ϵ and C_α encoding μ, δ, γ, ϵ and α heavy chain constant regions respectively (*Fig. 4*). During lymphocyte maturation, **two heavy chain gene rearrangements** occur. First a chosen D_H segment joins a J_H segment (**DJ joining**) and then the recombined D_HJ_H joins a chosen V_H segment (**VDJ joining**) (*Fig. 4*).

For the assembly of both heavy chain and light chain genes, the ends of the various DNA segments to be joined can also undergo modification during the recombination process and this modifies existing codons at these junctions or even creates new codons, thus increasing antibody diversity still further. In addition, antibody genes exhibit a higher than normal rate of mutation.

Class switching

When the complete heavy chain gene shown in *Fig. 4* is transcribed, it generates a heavy chain for an IgM antibody since it is always the first C segment after the recombined VDJ (in this case C_μ) that is transcribed. To switch to making a heavy chain for a different antibody class, say IgA, the lymphocyte DNA has to undergo yet another recombination event that moves C_α next to the recombined VDJ and deletes the intervening C segments. When this new gene is expressed,

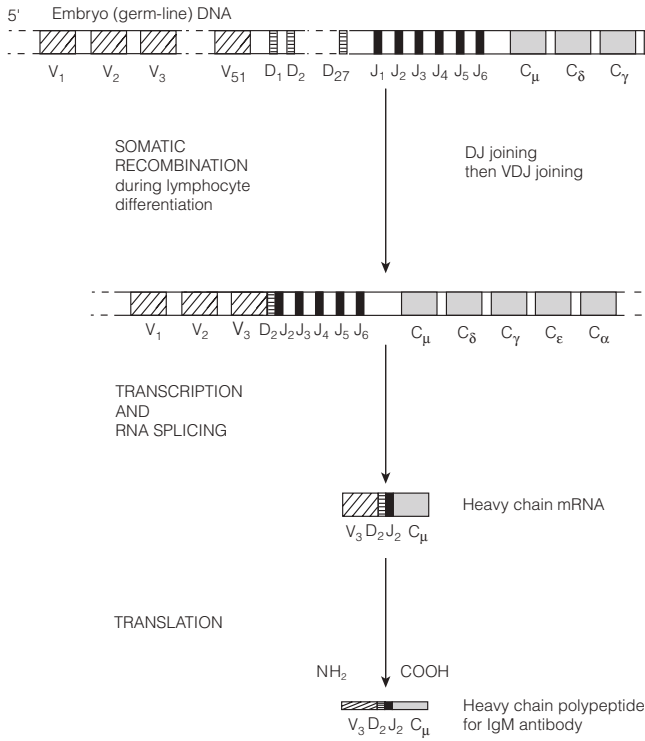

Fig. 4. Somatic recombination to create a human heavy chain gene and expression of that gene to produce heavy chain polypeptides. For simplicity, the constant regions for the various C_γ subclasses are not shown.

it will synthesize a C_α heavy chain for an IgA antibody instead of the earlier C_μ heavy chain for an IgM antibody. One crucial point about this **class switching** or **heavy chain switch** is that only the C region of the synthesized heavy chain changes; the variable region stays the same as before the switch. The specificity of the antibody is determined by the antigen-binding site, which is formed by the variable regions of the heavy and light chains and not by the constant regions. Thus even when class switching occurs so that the lymphocyte now makes IgA instead of IgM, the specificity of the antibody for antigens stays the same. The process of class switching at the DNA level (called **class switch recombination**) involves recombination between specific **switch sequences** located upstream of the heavy chain C segments (except C_δ).

D4 ANTIBODIES AS TOOLS

Key Notes

Immunolocalization methods

Because of the high specificity of an antibody for its epitope, an antibody raised against a particular protein antigen can be used to determine the location of that antigen in a cell using immunofluorescence light microscopy or immuno-electron microscopy.

ELISA

ELISA can be used to quantify the amount of a specific protein antigen in a sample. The antibody is bound to an inert polymer support, then exposed to the sample. Unbound protein is washed away and a second antibody that reacts with the antigen at a different epitope is added. The second antibody used is one that has an enzyme attached to it that converts a colorless or nonfluorescent substrate into a colored or fluorescent product. The amount of second antibody bound, and hence the amount of protein antigen present in the original sample, is determined by quantification of the intensity of color or fluorescence produced.

Immunoblotting

Protein samples are separated by one-dimensional SDS-PAGE or two-dimensional gel electrophoresis in polyacrylamide gels. The separated proteins are then transferred (blotted) to a nitrocellulose or nylon sheet. This is incubated with specific antibody to the protein and then unbound antibody is washed away. Those proteins in the gel that bind the antibody are detected either by autoradiography (if the specific antibody was radiolabeled) or by using a second labeled antibody that binds to the primary antibody.

Immunoaffinity chromatography

Immunoaffinity chromatography can be used to purify protein antigens by immobilizing the relevant antibodies on an inert matrix such as polysaccharide beads. When exposed to a protein mixture, only the protein recognized by that antibody will bind to the beads and can be eluted later in pure or almost pure form. Cells bearing the antigen on their surface can also be purified using a similar procedure.

Related topics

Bioimaging (A4)
Protein purification (B6)
Electrophoresis of proteins (B7)
The immune system (D1)
Antibodies: an overview (D2)

Immunolocalization methods

The availability of an antibody (immunoglobulin) against a specific antigen offers the opportunity to use that antibody in a range of immunological methods. The site recognized by an antibody on the antigen is called the **antigenic determinant** or **epitope**. The high specificity of an antibody for its epitope allows it to be used as a reagent for determining the location of the antigen in a cell (**immunocytochemistry**), for example by coupling a fluorescent label to the antibody and then using fluorescence to localize its sites of binding by **immuno-**

fluorescence light microscopy (see also Topic A4). Even higher resolution can be achieved using antibody to which electron-dense particles, such as ferritin or colloidal gold, have been coupled and then viewing the binding sites using electron microscopy (Topic A4). Indeed, **immuno-electron microscopy** can map the position of protein antigens within macromolecular complexes such as ribosomes.

ELISA

Specific antibodies can also be used to quantify the amount of the corresponding antigen in a biological sample. Several types of immunological assays exist. A popular version is **enzyme-linked immunosorbent assay (ELISA)** (see *Fig. 1*) which can readily detect and quantify less than a nanogram of a specific antigenic protein. In ELISA, the specific antibody is coupled to a solid support. A convenient format for ELISA is to use a plastic tray that has molded wells in it (a

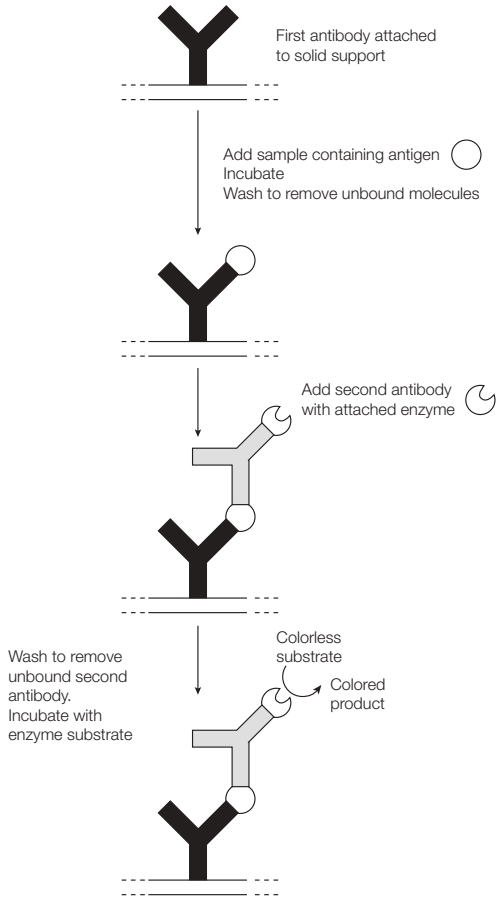

Fig. 1. ELISA using a second antibody with an attached (conjugated) enzyme that converts a colorless substrate to a colored product.

microtiter tray) where the antibody has been coupled to the plastic forming the wells. Samples to be assayed are added to the wells. If antigen is present that is recognized by the antibody, it becomes bound (*Fig. 1*). The wells are then washed to remove unbound protein and incubated with a second antibody that recognizes the protein but at a different epitope than the first antibody (*Fig. 1*). The second antibody is attached to an enzyme that can catalyze the conversion of a colorless or nonfluorescent substrate into a colored or fluorescent product. The intensity of the color or fluorescence produced for each sample is then measured to determine the amount of antigen present in each sample. Several machines are commercially available that scan the wells of microtiter plates following ELISA and quantify the amount of antigen bound in each well.

Immunoblotting

This technique can be used for detection of one or more antigens in a mixture. The sample is electrophoresed on an SDS–polyacrylamide gel (SDS-PAGE; see Topic B7) that separates the proteins on the basis of size, resulting in a series of protein bands down the gel (*Fig. 2*). Because the gel matrix does not let large

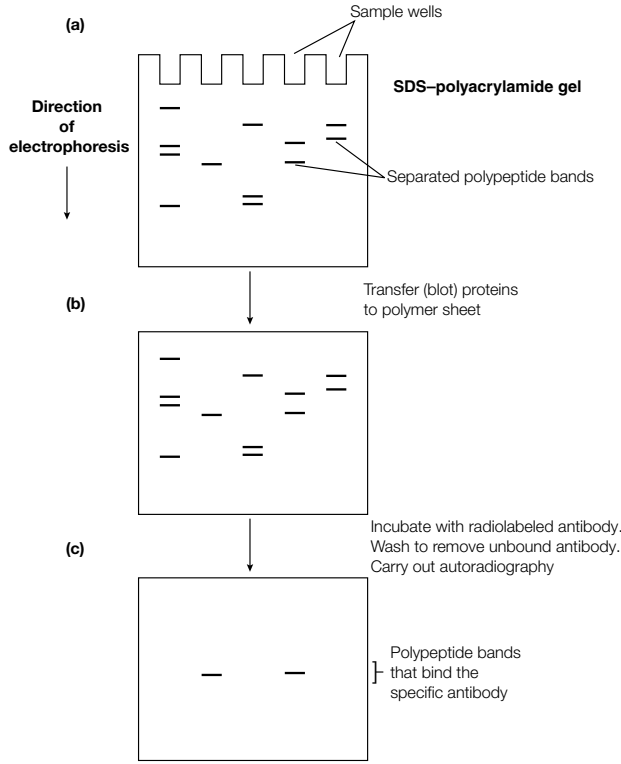

Fig. 2. Immunoblotting using a radiolabeled antibody (a) SDS polyacrylamide gel electrophoresis, yielding polypeptides separated into discrete bands; (b) nitrocellulose or nylon membrane onto which the protein bands have been blotted (i.e. a Western blot); (c) autoradiograph after incubating the Western blot with radiolabeled antibody, washing away unbound antibody and placing the membrane against X-ray film.

proteins such as antibodies enter readily, the sample proteins must be first transferred to a more accessible medium. This process is called **blotting**. The gel is placed next to a nitrocellulose or nylon sheet and an electric field is applied so that proteins migrate from the gel to the sheet where they become bound. This particular form of blotting (i.e. blotting of proteins) is called **Western blotting** (see also Topic B7) to distinguish it from blotting of DNA (Southern blotting; see Topic I3) or RNA (Northern blotting; see Topic I3). To detect specific proteins (antigens) using antibody probes (**immunoblotting**), the Western blot is incubated with a protein such as casein to bind to nonspecific protein-binding sites and hence prevent spurious binding of antibody molecules in subsequent steps. This step is said to 'block' nonspecific binding sites. The Western blot is then reacted with labeled antibody, unreacted antibody is washed away and those protein bands that have bound the antibody become visible and hence are identified (*Fig. 2*). The method of visualization depends on how the antibody was labeled. If the labeling is by the incorporation of a radiolabel (e.g. ^{125}I), then autoradiography is carried out to detect the radioactive protein bands (*Fig. 2*). Alternatively, the antibody may be detected by incubating the sheet with a second antibody that recognizes the first antibody (e.g. if the first specific antibody was raised in rabbits, the second antibody could be a goat anti-rabbit antibody). The second antibody could be radiolabeled and its binding detected by autoradiography or it could be conjugated to an enzyme that generates a colored product as in ELISA (see above). Immunoblotting can also be used to analyze specific antigens after two-dimensional gel electrophoresis which resolves proteins as spots, separated on the basis of both charge and size (see Topic B7).

Immunoaffinity chromatography

Immunoaffinity chromatography is one example of a range of different separation procedures generally called affinity chromatography which depend on high affinity interactions between two components (see Topic B6). In immunoaffinity chromatography, a specific antibody to a protein antigen may be coupled to an inert matrix such as polysaccharide beads. These can be placed in a column and the cell sample loaded on. The protein antigen will bind to the matrix but other components will flow through the column. The protein antigen can then often be eluted in pure, or almost pure form; well over 1000-fold purification is routinely achieved in this single step. If the protein antigen is normally exposed on the plasma membrane of a desired cell type, these cells can be purified from a cell mixture by passing the mixture through the column. Only cells bearing the antigen on their surface will bind and can be eluted subsequently.