7 Immunochemical techniques

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7.1 INTRODUCTION

The immune system of mammals has evolved over millions of years and provides an incredibly elegant protection system which is capable of responding to infective challenges as they arise. The system is fluid-based and both the cells of immunity and their products are transported throughout the body, primarily in the blood and secondarily through fluid within the tissues and organs themselves. All areas of the body are protected by immunity apart from the central nervous system including the brain and eyes. There are several cell types involved in immune responses, each with a role to play and each controlled by chemical mediators known as **cytokines**. This control is essential as the immune system is such a powerful tool it needs careful management to ensure its effective operation. Both over- and under-activity could have fatal consequences.

All vertebrates have advanced immune systems which show the similarities that you would expect from our common evolutionary past. The more advanced the

vertebrate the more complex the immune system. Fish and amphibians have fairly rudimentary immunity with the most sophisticated being found in mammals. The immune system is broadly additive; more complex animals have elements analogous to those found in primitive species but have extra features as well.

For the purposes of this chapter we will focus on the mammalian immune system although the use of birds for antibody production will be discussed in Section 7.1.2.

Immunity is monitored, delivered and controlled by specialised cells all derived from stem cells in the bone marrow. There are motile **macrophages** which move around the body removing debris and foreign materials, and two lineages of **lymphocyte**, B and T, which provide immediate killing potential but also provide the mechanism for the production of antibodies. There are also assorted other cells whose function is to rush to areas of the body where a breach of security has occurred and deliver potent chemicals capable of sterilising and neutralising any foreign bodies.

For mammalian immunity to function effectively it is vital that the cells of the system can recognise the difference between self and non-self. There are three ways in which this is achieved. Mammals have a pre-programmed ability to recognise and immediately act against substances derived from fungal and bacterial micro-organisms. This is mediated through a series of biological chemicals known as the **complement system** which are capable of adhering to and killing bacteria, fungi and some viruses. Secondly, the immune system is capable of recognising when a substance is close to but not quite the same as self. This is a response based on 'generic' circulating antibodies which are able to discriminate between self and non-self. Lastly, every individual has a unique 'signature' which is caused by a pattern of molecules on every cell surface. Cells of the immune system read this unique code and any cells differing from the authorised version are targeted and destroyed through a **T-cell**-mediated response. These three systems do not operate in isolation, they form a cohesive network of surveillance in which all of the cell types co-operate to provide the most appropriate response to any breach of security. In mammals the first line of defence against attack is the skin and any breaches of it are responded to by the cells of the immune system even though no foreign material is present. This response is mediated by cell messengers known as cytokines which can be released from damaged tissue or cells of the immune system near to the site of injury.

For the purposes of this chapter it is the antibody response in mammals that will be focussed on as these are the molecules that we are able to harness for our uses where a specific protein sequence or molecular structure has to be identified. As previously mentioned it is impossible to discuss one area of cellular immunity in isolation and so reference will be made to how the rest of the immune system contributes to the manufacture of antibodies by mammals.

An **antigen** is a substance capable of causing an immune response leading to the production of antibodies and they are also the targets to which antibodies will bind. Antibodies are antigen specific and will only bind to the antigen that initiated their production.

7.1.1 Development of the immune system

Mammalian embryos develop an immune system before birth which is capable of providing the newborn with immediate protection. Additional defences are acquired from maternal milk and this covers the period during which the juvenile immune system matures to deal with the requirements of the organism after weaning takes place. For an immune system to function effectively it must be organic in its ability to react to situations as they arise and the mammalian system has an extremely elegant way of dealing with this.

The cells of the mammalian immune system are descended from distinct lineages derived initially from stem cells, and those producing antibodies are known as **B cells** (also called B lymphocytes). These cells have the ability to produce antibodies which recognise specific molecular shapes. Cells of the immune system known as **macrophages**, **dendritic cells** and other **antigen-presenting cells** (APCs) have the ability to recognise 'foreign' substances (antigens) within the body and will attack and digest them when encountered. The majority of antigens found by the body are from viruses, bacteria, parasites and fungi, all of which may infect an individual. All of these organisms have proteins and other substances that will be **antigenic** (behave as an antigen) when encountered by the immune system. Organisms that infect or invade the body are known as **pathogens** and will have many antigens within their structures. The presenting cells process antigens into small fragments and present them to the B cells. The fragments contain **epitopes** which are typically about 15 amino acid residues in size. This size corresponds to the size that the antibody binding site can bind to. After ingesting the antigen fragments the B cells recruit 'help' in the form of cytokines from T cells which stimulates cell division and secretion. Each B cell that was capable of binding an antigen fragment and has ingested it will then model an antibody on the shape of an epitope and start to secrete it into the blood.

During embryonic development the immune system has to learn what is self and what is foreign. Failure to do this would lead to self-destruction or would lead to an inability to mount an immune response to foreign substances. During development this is achieved by selective **clonal deletion** (see Fig. 7.1) of self-recognising B cells. Early in the development of the immune system, B cell lineages randomly reassort the antibody-creating genes to produce a 'starter pack' of B cells that will respond to a huge number of molecular shapes. These cells have these randomly produced antibodies bound to their surface ready to bind should an antigen fit the antibody-binding site. These provide crude but instant protection to a large number of foreign substances immediately after birth. They also are the basis for the B cells that will provide protection for the rest of the animal's life. However, within the population of randomly produced B cells are a number which will be responsive to self-antigens, which are extremely dangerous as they could lead to the destruction of parts of the animal's body. Embryos are derived exclusively from cells derived from the fusion of egg and sperm. There are no cells derived from the mother within the embryonic sac in the uterus and so everything can be regarded as being immunologically part of 'self'. Any B cells that start to divide within the embryo prior to birth are responding to 'self' antigens and are destroyed as potentially dangerous. This selective clonal deletion is

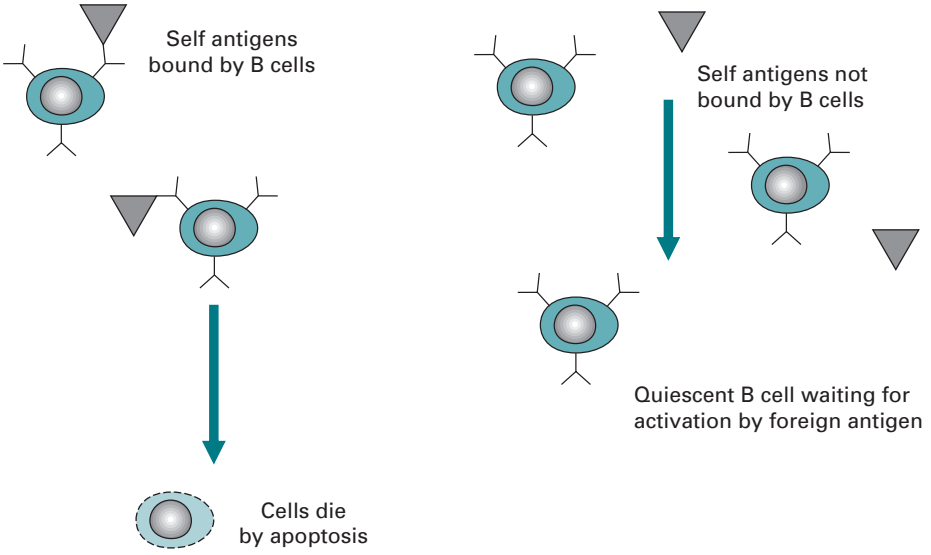

Fig. 7.1 Clonal deletion.

fundamental to the development of the immune system and without it the organism could not continue to develop. The remainder of the B cells that have not undergone cell division will only recognise non-self antigens and are retained within the bone marrow of the animal as a quiescent cell population waiting for stimulation from passing stimulated antigen-presenting cells. Stimulation of B cells requires both the presence of macrophages and also T lymphocytes. T cells are descended from an alternative lymphocyte lineage to the B cells and are responsible for 'helping' and 'suppressing' the immune response. T cells also undergo clonal selection during development to ensure that they do not recognise self antigens. In addition they are positively selected to ensure that they do recognise proteins of the **major histocompatibility complex** (MHC) found on cell surfaces. The balance of appropriate immune response is governed by the interplay of T and B lymphocytes along with macrophages and other antigen-presenting cells to ensure that an individual is protected but not endangered by inappropriate responses.

After birth, exposure to foreign materials will cause an immediate response resulting in antibody production and secretion by B cells. The antibody binds to the target and marks it as foreign and it is then removed by the body. Macrophages are responsible for much of the removal of foreign material which they ingest by **phagocytosis** (an uptake system that some cells use to transport particles from outside the surface membrane into the cell body). The material is then digested and exported to the cell surface as small fragments (antigens) which are then presented to passing B cells. Should a B cell carry an antibody that binds the antigen then it will take the antigen from the macrophages and this causes a number of intracellular changes known as **B cell activation**. B cell activation involves the recruitment of T cells which stimulates cell growth and metabolism. B cell activation may also occur without the presence of macrophages or other presenting cells when the lymphocyte is directly

exposed to antigens. The stimulation of the B cells leads to two major changes apart from antibody secretion. It leads to a larger population of cells being retained in the bone marrow that recognise the antigen. These are known as **memory cells** as they have the ability to recognise and rapidly respond should the antigen be encountered again. Binding antigen leads to cell division and antibody secretion but it also causes the cells to refine the quality of the antibody they produce. **Avidity** is the strength with which the antibody sticks to the antigen and **affinity** is the 'fit' of the antibody shape to the target. Both of these can be improved after the B cells have been stimulated but require more than one exposure to the antigen. The process is known as **affinity maturation** and is characterised by a change in antibody type from predominantly low-affinity pentameric (five molecules linked together) **immunoglobulin M** (IgM) to the high-affinity **immunoglobulin G** (IgG). Other antibody types may be produced in specific tissues and in response to particular antigens. For example parasites in the intestines often induce high levels of IgE in the gut mucosa (innermost layer of the gut which secretes large amounts of mucous). After several encounters with an antigen a background level of specific antibody will be found in the animal's blood along with a population of memory cells capable of rapidly responding to its presence by initiating high levels of antibody secretion. This status is known as **immune** and is the basis of both artificial **immunisations** for protection against disease and also for the production of antibodies for both diagnostic and therapeutic use.

7.1.2 Harnessing the immune system for antibody production

There are two major classes of antibodies used in immunochemistry: these are **polyclonal** and **monoclonal**. Polyclonal antibodies are produced in animals by injecting them with antigens. They are derived from the animal blood serum and their name means that they have been produced by many clones. This refers to the fact that the B cells that have made them will be producing antibodies to many different epitopes on the antigen and will involve the secretion of antibody by many B cell clones. Polyclonal antibodies are essentially a population of antibody molecules contributed by many B cell clones.

Monoclonal antibodies are produced by animal cells artificially in tissue culture, and as their name suggests the antibody produced comes from a single cell clone. The cells that make them are known as **hybridomas** and are produced from the fusion of a cancer cell line and B cells. Monoclonal antibodies are **epitope specific** whereas polyclonal antibodies are **antigen specific**. This difference is fundamental to the way in which they can be used for both diagnostics and therapeutics.

Mammals will produce antibodies to practically any foreign material that is introduced into their bodies providing it has a molecular weight greater than 5 000 Da. The only restriction to this is antigens that are closely related to substances found in the animal itself. Many mammalian proteins and other biochemical substances are highly conserved and are antigenically very similar in many species. This can lead to problems in producing antibodies for diagnostic and therapeutic use. The immune system is incapable of mounting a response to 'self' as discussed earlier and because of this, some antigens may not be able to produce an antibody response

in some species. Providing that the antigen is large enough and that it does not resemble proteins in the host animal then antibodies can be produced to a huge number of substances which can be used in all branches of diagnostics and therapeutics. There are three types of antibodies that can be produced: these are polyclonal, monoclonal and recombinant. Each of these antibody types has advantages but also limitations and should be viewed as complementary to each other as each has specific areas where they are particularly useful.

Polyclonal antibodies are produced in a number of animal species. Antibodies are generated by immunising the host with the substance of interest usually three or four times. Blood is collected on a number of occasions and the antibody fraction purified from the blood serum. The exception to this is chicken polyclonal antibodies which are harvested from eggs. Generally, larger animals are used since antibody is harvested from the blood of the animal and bigger volumes can be obtained from larger species. Historically, the first antibodies produced artificially for diagnostic purposes were polyclonal. They are the cheapest of antibodies to produce and have many uses in diagnostics. They have limited use in therapeutics, however, as there are problems in that they themselves can be antigenic when injected into other animals. There are exceptions to this and neutralising antibodies to snake venom and prophylactic (reducing risk of infection) antiviral injections fall into this category. Polyclonal antibodies are cheap to produce, robust but less specific than other antibodies and will have variable qualities depending on the batch and specific donor animal.

Monoclonal antibodies are secreted by mammalian cells grown in synthetic medium in tissue culture. The cells that produce them are known as hybridomas and are usually derived from donor mouse or rat lymphocytes. Human monoclonal antibodies are also available but they are produced by different methodologies to the rodent ones. The murine system was first described in 1976 when Kohler and Milstein published their work. Monoclonal antibodies have radically altered the possible uses for antibodies in both diagnostics and therapeutics. The basis of the technology is the creation of the hybridoma by fusing antibody-secreting B lymphocytes from a donor animal to a tumour cell line. B lymphocytes have a limited lifespan in tissue culture but the hybridoma has immortality conferred by the tumour parent and continues to produce antibody. Each hybridoma is derived from a single tumour cell and a single lymphocyte and this has to be ensured by **cloning**. Cell cloning is the process where single cells are grown into colonies, in isolation from each other so that they can be assessed and the best chosen for future development. Once cloned, the cell lines are reasonably stable and can be used to produce large quantities of antibody which they secrete into the tissue culture medium that they are grown in. The antibody they produce has the qualities that the parent lymphocyte had and it is this uniqueness that makes monoclonal antibodies so useful. During immunisation the B cells are presented with antigen fragments by macrophages and other antigen-presenting cells and each cell then produces a specific antibody to the fragment it has been presented with. The specific site that the antibody recognises is known as an **epitope** which is approximately 15 amino acids in size. There are thousands of potential epitopes on the antigen. The cell fusion process generates many

hundreds of hybridoma clones, each making an individual antibody. The most important part of making hybridomas is the screening process that is used to select those of value. Monoclonal antibodies are epitope specific and so it is important that the screening process takes this into account to ensure that antibodies selected have the correct qualities needed for the final intended use. They can be used for human and veterinary therapeutics although they are antigenic if used unmodified. Monoclonal antibodies can be processed to modify antigenicity to make them more useful in therapeutics. Mouse hybridomas can also be engineered so that the antibodies that they make have human sequences in them. These humanised antibodies have been used very successfully for treating a range of human conditions including breast cancer, lymphoma and the rejection symptoms after organ transplantation. Monoclonal antibodies are more expensive to produce than their polyclonal counterparts but have qualities that can make them more valuable. They are highly specific and reasonably robust but may be less avid than polyclonal antibodies. They are produced from established cell lines in tissue culture and should show little in the way of batch variation.

Recombinant antibodies are produced by molecular methodologies and are expressed in a number of systems, both prokaryotic and eukaryotic. Attempts have also been made to express antibodies in plants and this has had some success. The idea of producing antibody in crop plant species such as potato is very attractive as the costs of growing are negligible and the amounts of antibody produced could be very large.

Two basic methods can be used to produce recombinant antibodies. Existing **DNA libraries** can be used to produce **bacteriophage** expressing antibody fragments on their surface. Useful antibodies can be identified by assay and the bacteriophage producing it then used to transfect the antibody DNA into a prokaryotic host cell type. The antibody can then be produced in culture by the recombinant cells. The antibodies produced are monoclonal but do not have the full structure of those expressed by animals or cell lines derived from them. They are less robust and as they are much smaller than native antibodies it may not be possible to modify them without losing binding function. The great advantage of using this system is the speed with which antibodies can be generated, generally in a matter of weeks. Typically the timescale for producing monoclonal antibodies from cell fusions is about 6 months.

Antibodies can also be generated from donor lymphocyte (B cell) DNA. The highest concentration of B cells is found in the **spleen** after immunisation and so this is the tissue usually used for DNA extraction. The antibody-coding genes are then selectively amplified by **polymerase chain reaction** (PCR) and then **transfected** (inserted into DNA) into a eukaryotic cell line. Usually a resistance gene is co-transfected so that only recombinant cells containing antibody genes will grow in culture. The cells chosen for this work are often those most easily grown in culture and may be rodent or other mammalian lines. Chinese hamster ovary (CHO) cells are often used for this and have become the industry standard amongst biotechnology companies. Yeasts, filamentous algae and insect cells have all also been used as recipients for antibody genes with varying degrees of success.

7.1.3 Antibody structure and function

Antibodies as they are found in nature are all based on a Y-shaped molecule consisting of four polypeptide chains held together by disulphide bonds (see Fig. 7.2). There are two pairs of chains, known as heavy (H) and light (L); each member of the pair is identical. Functionally the base of the Y is known as the **constant region** and the tips of the arms are the variable region. The amino acid structure in the constant region is fairly fixed in an individual but varies between animal species. The amino acid structure in the variable region is composed of between 110 and 130 amino acids and it is variations in these that forms the different binding sites of the antibodies. The ends of both the heavy and light chains are variable and the antigen-binding site is formed by a combination of the two. The variable part of the antibody contains two further areas, the **framework** and **hypervariable** regions. There are three hypervariable and four framework regions per binding site. The hypervariable regions are structurally supported by the framework regions and form the area of direct contact with the antigen.

Antibodies can be fragmented by enzymatic degradation and the subunits produced are sometimes used to describe portions of the antibody molecule. Treatment with the enzyme papain gives rise to three fragments: two antigen-binding fragments (Fab) and one constant fragment (Fc). The enzyme digests the molecule at the **hinge region** and the resulting Fab fragments retain their antigen-binding capability. The Fc fragment has no binding region and has no practical use. Fab fragments are sometimes prepared and used for some immunochemical applications. Their smaller size may mean that they can bind to antigens in certain situations where the larger native molecule would have difficulty binding.

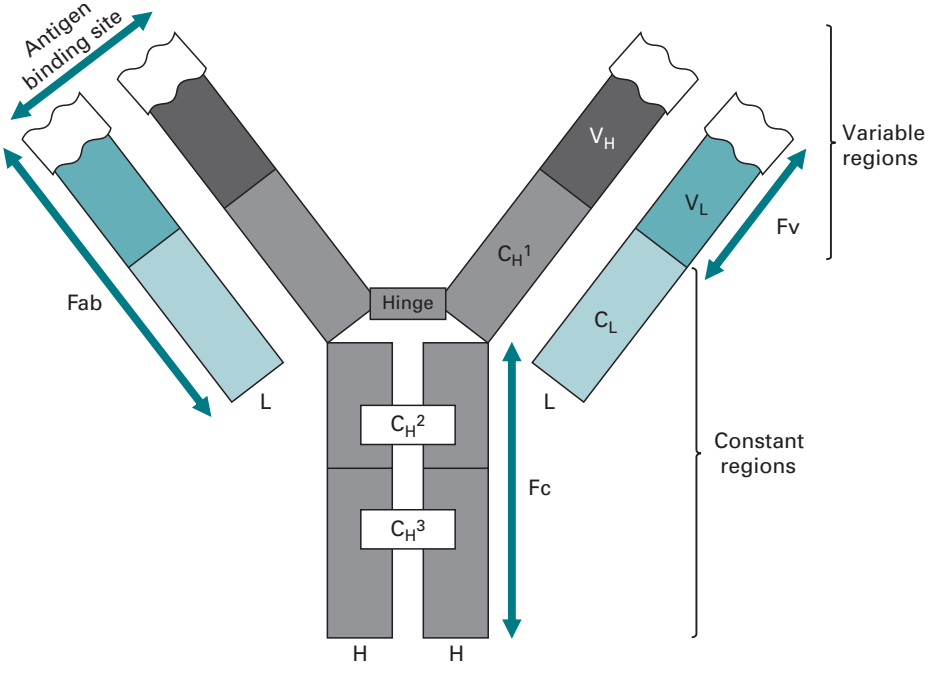

Fig. 7.2 Immunoglobulin G.

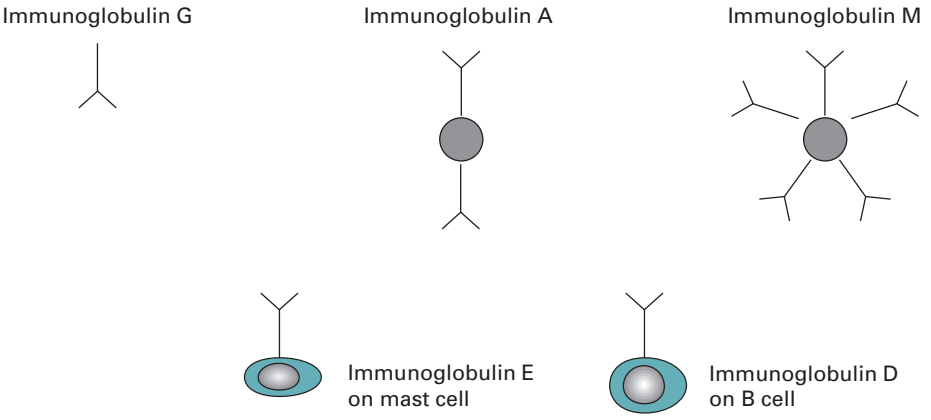

Fig. 7.3 Immunoglobulin classes.

Treatment with the enzyme papain gives rise to one double antigen-binding fragment **(F(ab′)$_2$)** and multiple Fc fragments. The enzyme digests the Fc until it reaches the hinge region of the antibody which is protected by a disulphide bond. F(ab′)$_2$ fragments have two binding sites and can be used in place of native antibody molecules.

There are five major classes of antibody molecule, also known as immunoglobulins (Ig); G is the commonest and it is characterised by its Y-shaped structure. The other classes of antibody are immunoglobulins M, A, D and E (Fig. 7.3).

Immunoglobulin M (IgM) is produced early in immune responses. It is produced by immature and newly activated B lymphocytes that have been exposed to an antigen for the first time. It is found on the surface of B cells frequently in association with **IgD**. Structurally it is formed from five immunoglobulin G molecules in a ring complexed by a mu chain. It may also be found as a hexamer without the mu chain. The molecule tends to have low affinity and poor avidity to antigen. It is much less specific and will react to a range of antigens without immunisation having taken place. It is known as 'natural antibody' as a result. Its production rises dramatically after first exposure to antigen and is characteristic of the **primary immune response**. It is generally only found in serum as its large size prevents it from crossing tissue boundaries. The pentameric form is particularly useful for complexing antigen such as bacteria into aggregates either for disposal or for further processing by the immune system. Cells secreting IgM can progress to IgG production, in time, if the animal is challenged again by the antigen. This progression to IgG production is known as **affinity maturation** and requires maturation of the cells to memory cell status. After several encounters with an antigen a background level of specific antibody will be found in the animal's blood along with a population of B memory cells capable of rapidly responding to its presence by initiating high levels of antibody secretion. This status is known as immune and is the basis of both artificial immunisations for protection against disease and also for the production of antibodies for both diagnostic and therapeutic use. A status of hyperimmunity may be reached after repeated exposure to an antigen leading to extremely high levels of circulating

antibody. Hyperimmunity carries risk, as additional exposure to antigen can lead to anaphylactic shock due to the overwhelmingly large immune response. Conversely, a total loss of immune response to an antigen can occur after repeated immunisation as a state of immune tolerance to the antigen is reached. Acquired immune tolerance is a response to overstimulation by an antigen and is characterised by a loss of circulating B cells reactive to the antigen and also by a loss of T cell response to the antigen. This can be used therapeutically to protect individuals against allergic responses.

Immunoglobulin A (IgA) is a dimeric form of immunoglobulin essentially with two IgG molecules placed end to end with the binding sites facing outwards. They are complexed with a J chain. It is predominantly found in secretions from mucosa and is resistant to enzyme degradation due to its structure. It is primarily concerned with protection of the mucosal surface of the mouth, nose, eyes, digestive tract and genito-urinary system. It is produced by B cells resident in the mucosa and is directly secreted into the fluids associated with the individual tissues. It is of little use in immunochemistry as it cannot be purified easily and is prone to spontaneous aggregation. Occasionally a hybridoma is derived secreting antibodies with this isotype and it may be that this is the only source of a rare antibody. In this case an indirect assay may be developed using the tissue culture supernatant derived from the hybridoma along with a specific anti-IgA antibody–enzyme conjugate.

Immunoglobulin D (IgD) is an antibody resembling IgG and is found on the surface of immature B cells along with IgM. It is a cellular marker which indicates that an immature B cell is ready to mount an immune response and may be responsible for the migration of the cells from the spleen into the blood. It is used by the macrophages to identify cells to which they can present antigen fragments.

Immunoglobulin E (IgE) also resembles IgG structurally and is produced in response to allergens and parasites. It is secreted by B lymphocytes and attaches itself to the surface of specialised cells known as **mast cells**. Exposure to allergen and its subsequent binding to the IgE molecules on the cell surface cause the antibodies to cross-link and move together in the cell membrane. This cross-linking causes the cell to degranulate releasing **histamine**. Histamine is responsible for the symptoms suffered by individuals as a result of exposure to allergens.

Immunoglobulin G and to a lesser extent IgM are the only two antibodies that are of practical use in immunochemistry. IgG is the antibody of choice used for development of assays as it is easily purified from serum and tissue culture medium. It is very robust and can be modified by labelling with marker molecules (see Section 7.4) without losing function. It can be stored for extended periods of time at 4 °C or lower. Occasionally antigens will not generate IgG responses *in vivo* and instead IgM is produced. This is caused by the antigen being unable to activate the B cells fully and as a result no memory cells being produced. Such antigens are often highly **glycosylated** and it is the large number of sugar residues that block the full activation of the B cells. IgM can be used for assay development but is more difficult to work with. IgM molecules tend to be unstable and are difficult to label without losing function as the binding sites become blocked by the proximity of each other. This is known as **stearic hindrance**. They can be used directly from cell tissue culture supernatant in assays with an appropriate secondary anti-IgM enzyme conjugate.

7.2 MAKING ANTIBODIES

All methods used in immunochemistry rely on the antibody molecule or derivatives of it. Antibodies can be made in various ways and the choice of which method to use is very much dependent on the final assay format. For an antibody to be of use it has to have a defined specificity, affinity and avidity as these are the qualities that determine its usefulness in the method to be used. There are considerable cost differences in producing the various antibody types and it is important to remember that the most expensive product is not always the best.

7.2.1 Polyclonal antibody production

Polyclonal antibodies are raised in appropriate donor animals, generally rabbits for smaller amounts and sheep or goats for larger quantities. Occasionally rats or mice can be used for small research quantities of antibody. It is important that animals are sourced from reputable suppliers and that they are housed and managed according to domestic welfare legislation.

Usually antigens are mixed with an appropriate **adjuvant** prior to immunising the animals. Adjuvants are substances which increase the immunogenicity of the antigen and are used to reduce the amount of antigen required as well as stimulate specific immunity to it. Adjuvants may be chemicals such as detergents and oils or complex proprietary products containing bacterial cell walls or preparations of them. Pre-immune blood samples are taken to provide baseline IgG levels (Fig. 7.4). Immunisations are spaced at intervals to maximise antibody production usually at least 4–6 weeks apart although the first two may be given within 14 days. Blood samples are taken 10 days after the immunisation programme is complete and the serum tested for specific activity to antigen by a method such **enzyme-linked immunosorbent assay (ELISA)** (see Section 7.3). Usually a range of doubling serum dilutions are made (1/100–1/12 800) and tested against the antigen. Serum from a satisfactory course of immunisations will detect antigen at 1/6400 dilution indicating high levels of circulating antibody. Once a high level of circulating antibody is detected in test bleeds then donations can be taken. Animal welfare legislation governs permissible amounts and frequency of bleeds. Donations can be taken until the antibody titre begins to drop and if necessary the animal can be immunised again and a second round of donations taken.

Blood donations are allowed to clot and the serum collected. Individual bleeds may be kept separate or pooled to provide a larger volume of standard product. Serum can be stored at 4 °C or lower for longer periods.

It is also possible to produce antibodies in chicken eggs. Avian immunoglobulin is known as **immunoglobulin Y (IgY)** and chickens secrete it into eggs to provide protection for the developing embryo. This can be utilised for effective polyclonal antibody production. The chickens are immunised three or four times with the antigen and the immune status monitored by test bleeds. Eggs are collected and can yield up to 50 mg antibody per yolk. The antibody has to be purified from the egg yolks prior to use and a number of proprietary kits can be used to do this. Occasionally antigens that give a poor response in mammals can give much higher yields in chickens.

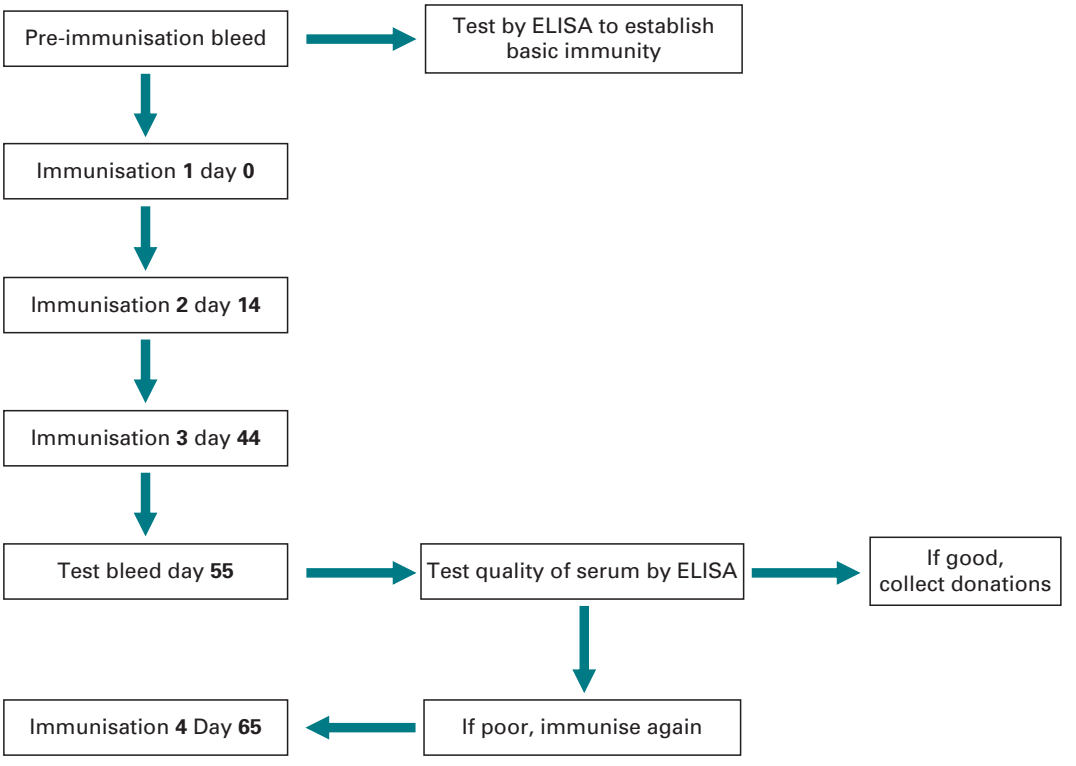

Fig. 7.4 Immunisation schedule.

Small quantities of very pure polyclonal antibodies can be produced in rats and mice in **ascitic fluid**. Ascites is a mammalian response to a tumour within the peritoneum (cavity containing the intestines). Fluid similar to plasma is secreted into the cavity of the animal and contains very high levels of the antibodies that the animal is currently secreting in its blood. Animals are immunised with the antigen of interest and once a high serum level is detected then ascitic fluid production is induced. Non-secretory myeloma cells such as NS-0 are introduced into the peritoneal cavity of the animal by injection and allowed to grow there. The presence of the tumour cells causes the animal to produce ascitic fluid which contains high levels of immunoglobulins to the original antigen. The fluid is removed by aspiration with a syringe and needle usually on three or four occasions over a month or so.

7.2.2 Monoclonal antibody production

Mice are usually the donor animal of choice for monoclonal antibody production although rats and other rodent species may be used. They are cheap to buy and house, and easy to manage and handle. The limitation on using other species is the availability of a suitable tumour partner for performing fusions. Balb/C is the usual mouse strain used for monoclonal antibody production and most of the tumour cell lines used for fusion are derived from this mouse. Females are usually used as they can be housed together without too much aggression.

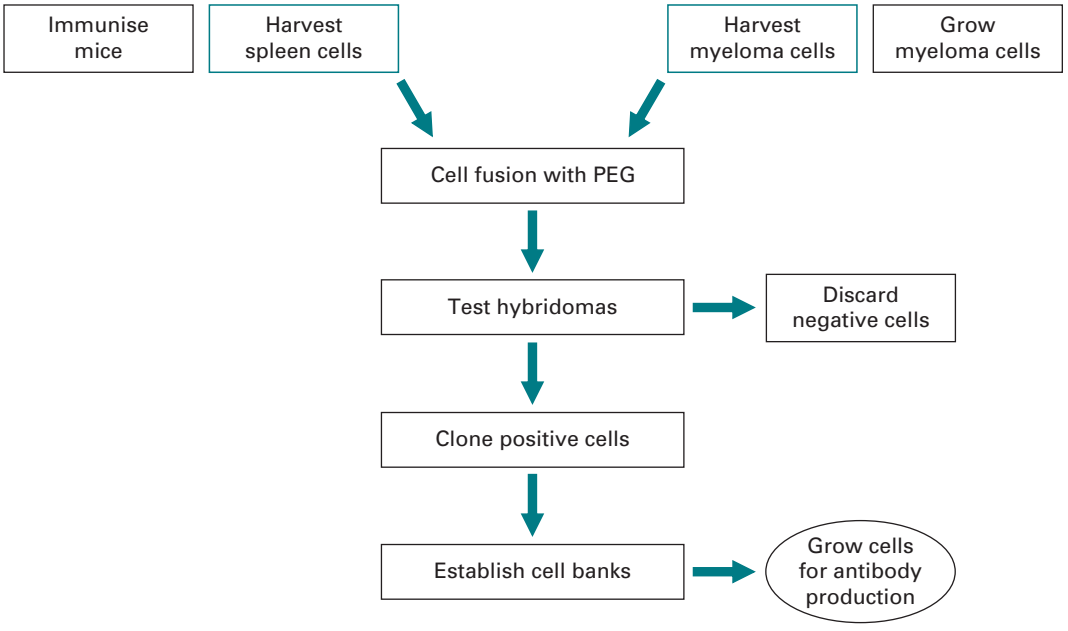

Fig. 7.5 Monoclonal antibody production.

Mice are immunised, usually three or four times over the course of 3–4 months, by the intraperitoneal route using antigen mixed with an appropriate adjuvant (Fig. 7.5). Test bleeds can be taken to monitor the immune status of the animals. Once the mice are sufficiently immune they are left for 2–3 months to 'rest'. This is important as the cells that will be used for the hybridoma production are memory B cells and require the rest period to become quiescent.

Mice are sacrificed and the spleens removed; a single spleen will provide sufficient cells for two or three cell fusions. Three days prior to cell fusion the partner cell line NS-0 is cultured to provide a log phase culture. If rat hybridomas are to be made then the fusion partner Y3 or its derivative Y0 can be used. Cell fusions can be carried out by a number of methods but one of the most commonly used is fusion by centrifugation in the presence of polyethylene glycol (PEG). Then 26×10^6 cells of spleen and fusion partner are mixed together in a centrifuge tube. A quantity of PEG is added to solubilise the cell membranes and the fusion carried out by gentle centrifugation. The PEG is removed from the cells by dilution with culture medium and the cells placed into 96-well tissue culture plates at a cell density of 10×10^5 per well. From experience, these cell numbers will produce only a single recombinant cell capable of growth in each well. Fusion partners are required to have a defective enzyme pathway to allow selection after cell fusion. NS-0 lacks the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) which prevents it from using a nucleoside salvage pathway when the primary pathway is disabled by the use of the antibiotic aminopterin. The tissue culture additive HAT which contains hypoxanthine aminopterin and thymidine is used to select for hybridomas after cell fusion. They inherit an intact nucleoside salvage pathway from the spleen cell parent which allows

them to grow in the presence of aminopterin. Unfused NS-0 cells are unable to assimilate nucleosides and die after a few days. Unfused spleen cells are unable to divide more than a few times in tissue culture and will die after a few weeks. Two weeks after the cell fusion the only cells surviving in tissue culture are hybridomas. The immunisation process ensures that many of the spleen cells that have fused will be secreting antibody to the antigen; however this cannot be relied upon and rigorous screening is required to ensure that the hybridomas selected are secreting an antibody of interest. Screening is often carried out by ELISA but other antibody assessing methods may be used. It is important that hybridomas are assessed more than once as they can lose the ability to secrete antibody after a few cell divisions. This occurs as chromosomes are lost during division to return the hybridoma to its normal chromosome quota.

Once hybridomas have been selected they have to be cloned to ensure that they are stable. Cloning involves the derivation of cell colonies from individual cells grown isolated from each other. In **limiting dilution cloning**, a cell count is carried out and dilutions of cells in media made. The aim is to ensure that only one cell is present in each well of the tissue culture plate. The plates are incubated for 7 days and cell growth assessed after this time. Colonies derived from single cells are then tested for antibody production by ELISA. It is essential to clone cell lines to ensure that they are truly monoclonal. It is desirable that a cell line should exhibit 100% cloning efficiency in terms of antibody secretion but some cell lines are inherently unstable and will always produce a small number of non-secretory clones. Providing such cell lines are not subcultured excessively then the problem may not be too great although it is usual to reclone these lines regularly to ensure that cultures are never too far from an authenticated clone.

It is very important to know the antibody isotype of the hybridomas as discussed previously and a number of commercial kits are available to do this. Most are based on lateral flow technology which will be discussed later in this chapter. Once the isotype of the antibody is established and it is clonally stable then cultures can be grown to provide both **cell banks** and antibody for use in testing or for reagent development.

Record-keeping is absolutely vital so that the pedigree of every cell line is known. It is also very important to be vigilant in handling and labelling flasks to prevent cross-contamination of cell lines. It is usual to name cell lines and use the clone and subclone number as part of the name. One such naming system used is: <fusion number>/<clone number>•<sub-clone number>•<additional sub-sub-clone numbers>. Other naming systems are used and it is up to the individual to find one that suits them best.

7.2.3 Freezing cells

Cell lines are frozen to provide a source of inoculum for future cultures. Cells cannot be grown indefinitely in culture as the required incubator space would be impractical in most tissue culture laboratories. Additionally, although established cell lines should be stable it is known that long-term culture leads to cellular instability and the increased risk of cellular change. Cells stored at the low temperatures achieved using

liquid nitrogen vapour are stable for many years and can be resuscitated successfully after decades. Cells are transferred into a specialist medium prior to freezing to protect them both as the temperature is lowered and also as the temperature is raised when thawed. Serum containing 10% DMSO works well as a freezing medium although serum-free media can be used if required. Cells must be in perfect health and in log phase prior to freezing. A typical freezing should contain around 1×10^6cells and this can be assessed by performing a cell count using a counting chamber. A confluent 25-cm^2 tissue culture T flask will contain approximately this many cells and for many applications it may not be necessary to carry out a cell count. The cells are harvested from the flask by tapping to dislodge them and pelleted by centrifugation to remove the culture medium. The cells are then resuspended in 1.0 ml freezing medium chilled to 4 °C, placed into a cryogenic vial and transferred to a cell freezing container. The freezing container contains butan-1-ol which when placed into a −70 °C freezer controls the rate of freezing to 1 °C per minute. The gradual freezing is necessary so that as the ice forms within the cells it does so as a glass and not as crystals which would expand and damage the cell structure. The cells are left for a minimum of 24 h and a maximum of 72 h prior to transfer into cryogenic storage. Transfer to liquid nitrogen storage must be rapid to prevent thawing of the cells. It is imperative that the vials are permanently marked and that the storage locations within the cryogenic vessel are noted for future retrieval.

7.2.4 Cell banking

Cell banks are established from known positive clones and are produced in a way that maximises reproducibility between frozen cell stocks and minimises the risk of cellular change (Fig. 7.6). A positive clone derived from a known positive clone is rapidly expanded in tissue culture until enough cells are present to produce 12 vials of frozen cells simultaneously. This is the **master cell bank** and is stored at −196 °C under liquid nitrogen vapour. The **working cell bank** is then derived from the master cell bank. One of the frozen vials from the working cell bank is thawed and rapidly grown until there are enough cells to produce 50 vials of frozen cells simultaneously. This is the working cell bank and it is also stored at −196 °C under liquid nitrogen vapour. This strategy ensures that all of the vials of the working cell bank are identical. All of the vials of the master cell bank are also identical and if a new working bank is required then it can be made from another vial from the master.

Cell banks work well if managed correctly but record-keeping is vital for their operation. A cell bank derived in this way will provide 550 working vials before the process of deriving a new master cell bank is required. If a new master cell bank is required this is produced by thawing and cloning from the last master cell bank vial and selecting a positive clone for expansion.

7.2.5 Antibodies to small molecules

The immune system will recognise foreign proteins and peptides providing that they have a molecular weight (mw) greater than about 2 000 Da (although above 5 000 Da is

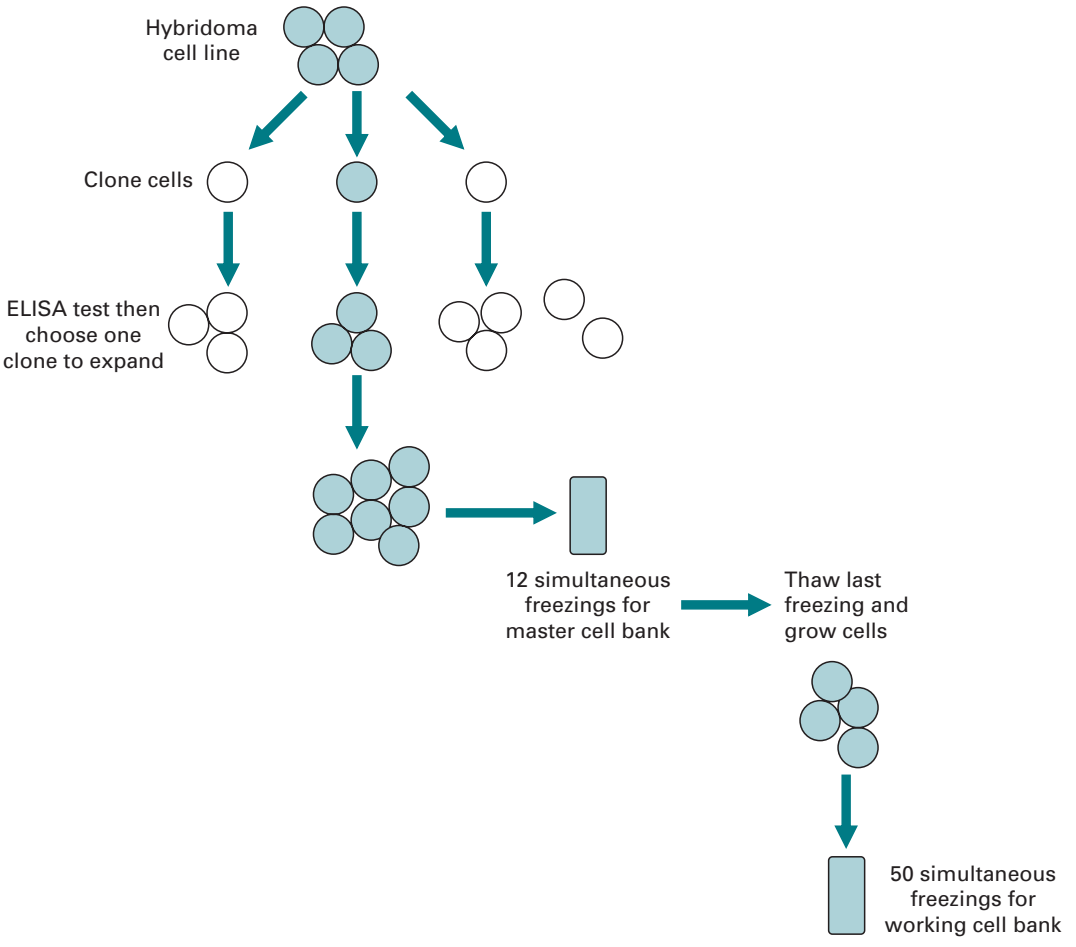

Fig. 7.6 Master and working cell banks.

optimal). The magnitude of the response will increase the greater the molecular weight. If an antibody is to be made to a molecule smaller than 2 000 Da then it has to be conjugated to a carrier molecule to effectively increase its size above the threshold for immune surveillance. These small molecules are known as **haptens** and may be peptides, organic molecules or other small chemicals. They are usually conjugated to a protein such as albumin, keyhole limpet haemocyanin or thyroglobulin and then used to immunise animals for antibody production (Fig. 7.7). If a polyclonal antibody is being made it is advisable to change the carrier protein at least once in the immunisation procedure as this favours more antibody being made to the hapten and less to each of the carrier proteins. If a monoclonal antibody is being made then the carrier protein can be the same throughout the immunisations. When screening hybridomas for monoclonal antibody production it is necessary to screen against the hapten and carrier separately. Any antibodies responding to both should be discarded as these will be recognising the junction between the hapten and carrier and will not recognise the native hapten.

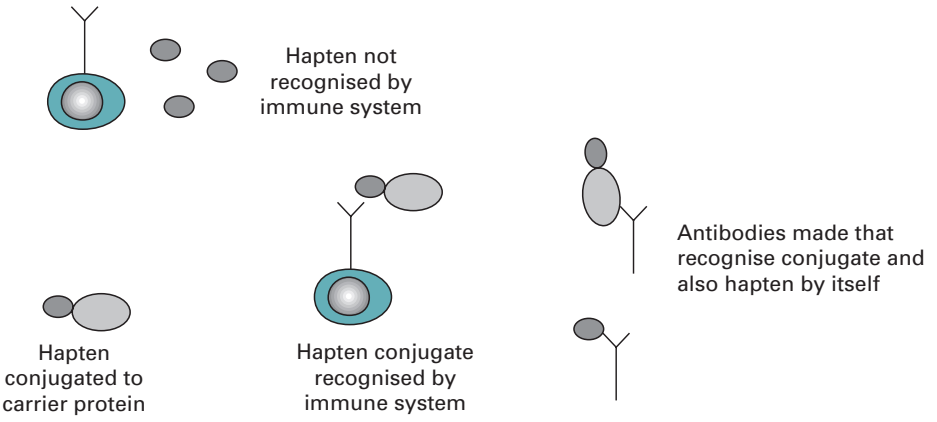

Fig. 7.7 Making antibodies to haptens.

7.2.6 Anti-idiotype antibodies

The binding site of an anti-idiotype antibody is a copy of an epitope. They are made by deriving a primary monoclonal antibody to the epitope of interest, usually a cell membrane receptor or other important binding site. These primary antibodies are then themselves used as antigen to produce secondary antibodies, some of which will recognise the binding site on the primary antibody. These are the anti-idiotype antibodies and they have the unique quality that their binding site structurally resembles the original epitope. They themselves can be used as vaccines as the immune response raised to them will cross-react with the native original epitope. Some human cancers have cell surface receptors that are unique to them and these can be used as a target for antibody therapy. Anti-idiotype antibodies raised to the cell receptors are used to immunise the patient. The resulting antibodies made by the patient bind to the receptors on the tumour cells allowing the immune system to recognise and destroy the tumour. The method has had some success in the treatment of ovarian and bowel tumours.

7.2.7 Phage display for development of antibody fragments

Bacteriophage or phage, as they are known, are viruses that infect and replicate within bacteria. They can be engineered by molecular methods to express proteins and providing the protein sequence is tagged to the coat protein gene then the foreign protein will be expressed on the virus surface. It is possible to isolate the variable (V) antibody coding genes from various sources and insert these into the phage resulting in single-chain antigen-binding (scFv) fragments. Whole antibodies are too large and complex to be expressed by this system but the scFv fragments can be used for diagnostic purposes. The DNA used in this process may come from immunised mouse B cells or from libraries derived from naive mouse (or other species). The V genes are cloned into the phage producing a library which is then assessed for specific activity. It is important to isolate clones that have the specific activity that is required and this can be done by immobilising the antigen onto a solid surface and then adding phage

clones to the immobilised antigen. The clones that bind to the antigen are desirable and those that do not bind are washed away. This technique is known as **panning** and refers to the technique used by nineteenth-century gold prospectors who washed gravel from rivers, using shallow pans that retained gold fragments. Once clones have been selected for antibody expression they can be multiplied in their host bacterium in liquid culture. The scFv fragments can be harvested using proprietary extraction kits and used to develop ELISA and other immunoassays.

7.2.8 Growing hybridomas for antibody production

Cell growth and storage is carried out for the development of cell banks but hybridomas are primarily grown for their products, monoclonal antibodies. All monoclonal antibodies are secreted into the tissue culture media that the cells are growing in. There are a number of ways that cells can be grown to maximise antibody yield, reduce media costs and simplify purification of the product from tissue culture medium. The simplest method for antibody production is static bulk cultures of cells growing in T flasks. T flasks are designed for tissue culture and have various media capacities and cell culture surface areas. For most applications a production run is between 250 ml and 1000 ml medium. Most cell lines produce between 4 and 40 mg of antibody per litre so the size of the production run is based on requirement. The cells from a working cell bank vial are thawed rapidly into 15 ml medium containing 10% foetal bovine serum and placed in an incubator at 37 °C supplemented with 5% CO_2. Once cell division has started, the flask sizes are increased using medium supplemented with 5% foetal bovine serum until the desired volume is reached. Once the working volume has been achieved the cells are left to divide until all nutrients are utilised and cell death occurs. Usually the timespan for this is around 10 days. The cell debris can then be removed by centrifugation and the antibody harvested from the tissue culture medium. For some applications the antibody can be used in this form without further processing.

Monoclonal antibodies can also be produced in ascitic fluid in mice. As described previously, cells can be grown in the peritoneal cavities of mice. **Nude mice** have no T cells and because of this have poor immune systems. They are often used for ascitic fluid production as they do not mount an immune response to the implanted cells. The mice should not be immunised prior to use as it is important that the only antibody present in the ascitic fluid is derived from the implanted cells. Hybridoma cells are injected into the peritoneum of the mice and allowed to grow there. These cells are secretory and produce high levels of monoclonal antibody in the ascitic fluid. The fluid is harvested by aspiration with a syringe and needle.

A number of *in vitro* **bioreactor** systems have been developed to produce high yields of monoclonal antibody in small volumes of fluid which mimics ascitic fluid production (Fig. 7.8). All of them rely on physically separating the cells from the culture medium by semipermeable membrane which allows nutrient transfer but prevents monoclonal antibody from crossing. The culture medium can be changed to maximise cell growth and health, and fluid can be removed from around the cells to harvest antibody. Some are based on a rotating cylinder with a cell-growing compartment at

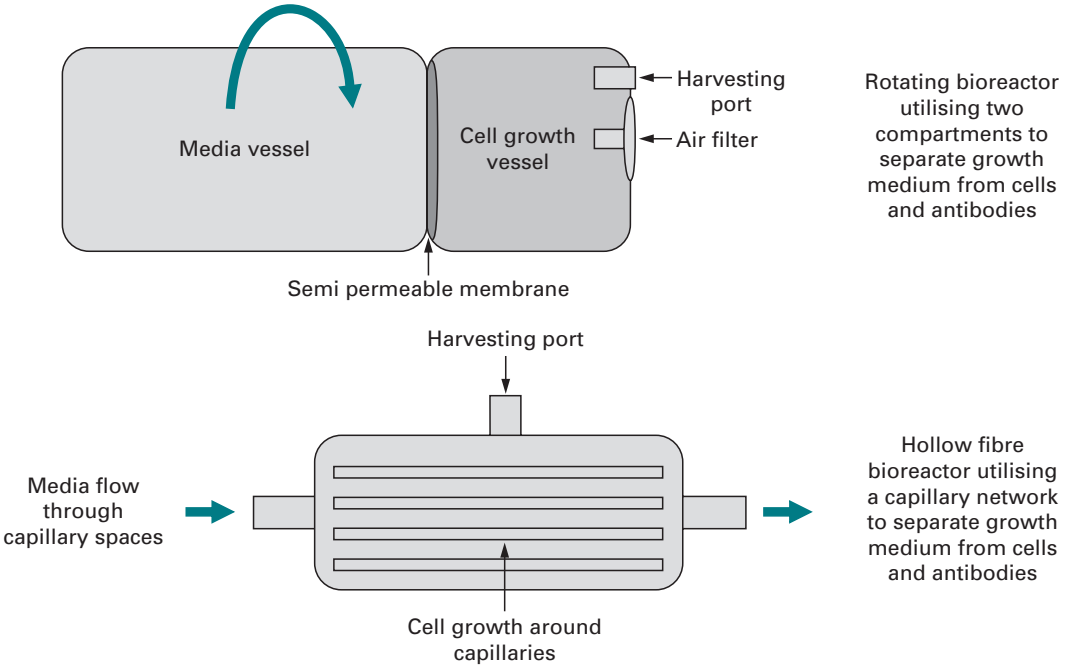

Fig. 7.8 Bioreactors for antibody production.

one end separated from the media container by a membrane. Others have capillary systems formed from membrane running through the cell culture compartment and in these the media is pumped through the cartridge to facilitate nutrient and gas exchange. These systems do produce high yields of antibody but can be problematical to set up and run. They are ideal where large quantities of monoclonal antibody are needed and space is at a premium. They are however prone to contamination by yeasts and great care must be exercised when handling them. Cells are grown in bioreactors for up to 6 weeks so the clone used must be stable and it is advisable to carry out studies on long-term culture prior to embarking on this form of culture. The major advantage of bioreactor culture is that the antibody is produced in high concentration without the presence of media components making it easy to purify. Total quantities per bioreactor run may be several hundred milligrams to gram quantities.

7.2.9 Antibody purification

The choice of method used for the purification of antibodies depends very much on the fluid that they are in. Antibody can be purified from serum by the addition of **chaotropic ions** in the form of saturated ammonium sulphate. This preferentially precipitates the antibody fraction at around 60% saturation and provides a rapid method for IgG purification. This method does not work well in tissue culture supernatant as media components such as ferritin are co-precipitated. Ammonium sulphate precipitation may be used as a preparatory method prior to further chromatographic purification.

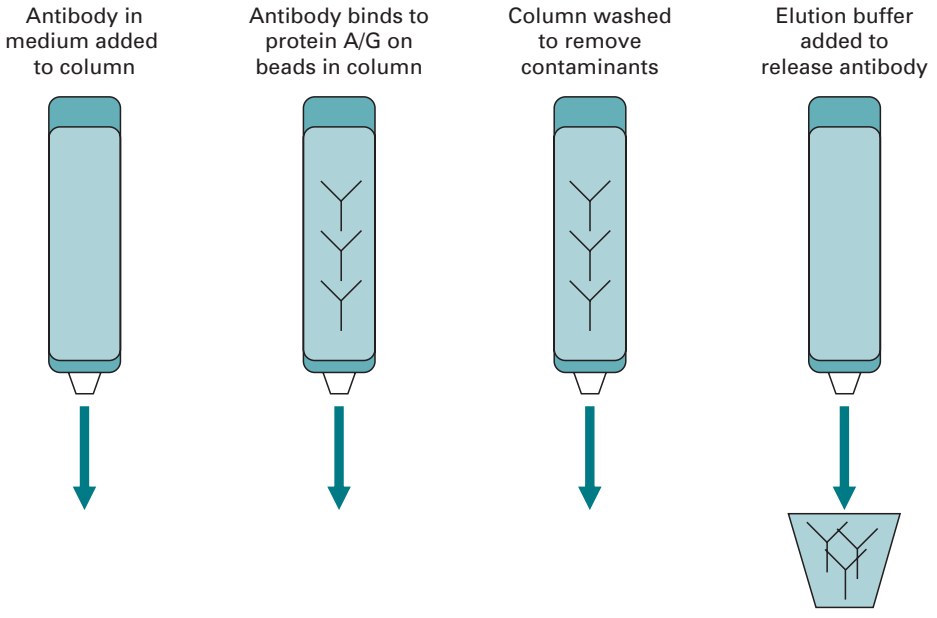

Fig. 7.9 Affinity chromatography.

Tissue culture supernatant is often concentrated before purification to reduce the volume of liquid. **Tangential flow** devices and **centrifugal concentrators** may be used to reduce the volume to 10% of the starting amount. This makes antibody purification by **affinity chromatography** much easier with the smaller volume of liquid (Fig. 7.9).

Antibodies from both polyclonal and monoclonal sources can be purified by similar means. In both cases the antibody type is IgG which allows purification by **protein A/G** affinity chromatography. Proteins A and G are derivatives of bacterial cells and have the ability to reversibly bind IgG molecules. Binding to the column occurs at neutral pH and the pure antibody fraction can be eluted at pH 2.0. Fractions are collected and neutralised back to pH 7.0. Antibody-containing fractions are identified by spectrophotometry using absorbance at 280 nm (specific wavelength for protein absorbance) and are pooled. A solution of protein at 1 mg cm^{-3} will give an absorbance reading of 1.4 at 280 nm. This can be used to calculate the amount of antibody in specific aliquots after purification.

Purified antibody should be adjusted to 1 mg cm^{-3} and kept at 4 °C, or −20 °C for long-term storage. It is usual to add 0.02% sodium azide to the antibody solution as this increases shelf-life by suppressing the growth of adventitious microorganisms. Antibodies can be stored for several years at 4 °C and for decades if kept below −20 °C without losing activity.

7.2.10 Antibody modification

Antibodies can be labelled for use in assays such as ELISA by the addition of marker enzyme such as **horse radish peroxidise** (HRP) or **alkaline phosphatase** (AP). Other enzymes such as urease have been used but HRP and AP are by far the most

popular. Linkage is achieved by simple chemistry to provide stable antibody–enzyme conjugates. Glutaraldehyde is a cross-linking compound and conjugation to HRP is carried out in two stages. Firstly the glutaraldehyde is coupled to reactive amino groups on the enzyme. The HRP–glutaraldehyde is then purified by **gel permeation chromatography** and added to the antibody solution. The glutaraldehyde reacts with amino groups on the antibody forming a strong link between the antibody and HRP. Alkaline phosphatase can be linked to antibody by glutaraldehyde using a one-step conjugation. The linkage is achieved through amino groups on the antibody and on the enzyme coupled with the glutaraldehyde.

A number of proprietary labelling reagents are also available for making antibody enzyme conjugates.

Fluorescent labels can also be added for use in immunofluorescent assays; usually **fluorescein** is the molecule of choice. **Fluorescein isothiocyanate** (FITC) is often the derivative used to label antibodies. FITC is a fluorescein molecule with an isothiocyanate reactive group (–N=C=S) replacing one of the hydrogens. This derivative is reactive towards primary amines on proteins and will readily react with antibodies to produce fluorescent conjugates.

It is also possible to link antibodies to gold particles for use in **immunosorbent electron microscopy** (ISEM) and **lateral flow devices**. Gold particles are prepared by citrate reduction of auric acid. The size of particle is predictable and can be controlled by pH manipulation. The gold particles are reactive and will bind antibodies to their surface forming **immunogold**. The immunogold particles are stable and can be stored at 4 °C until required.

Rare earth lanthanides can also be used as labels and have the advantage that a single assay can be used to detect two or three different antibody bindings. The lanthanides are attached to the antibodies as a chelate. The commonest of the chelating compounds used is diethylenetriamine pentaacetate (DTPA). Each lanthanide fluoresces at a different light frequency and so multiple assays can be carried out and the individual reactions visualised by the use of a variable wavelength spectrophotometer. Antibodies can also be attached to latex particles either by passive absorption or to reactive groups or attachment molecules on the surface of the latex. These can be used either as the solid phase for an immunoassay or as markers in lateral flow devices. Magnetised latex particles are available allowing the easy separation of latex particle/antibody/antigen complex from a liquid phase. Latex and magnetic particles may be purchased which have protein A covalently attached to their surface. Protein A binds the Fc portion of the antibody which orientates the molecules with the binding sites facing outwards.

7.3 IMMUNOASSAY FORMATS

The first immunoassay formats described were methods based on the **agglutination** reaction (Fig. 7.10). The reaction between antibody and antigen can be observed when agglutination occurs and is characterised either by a gel formation in a liquid phase or

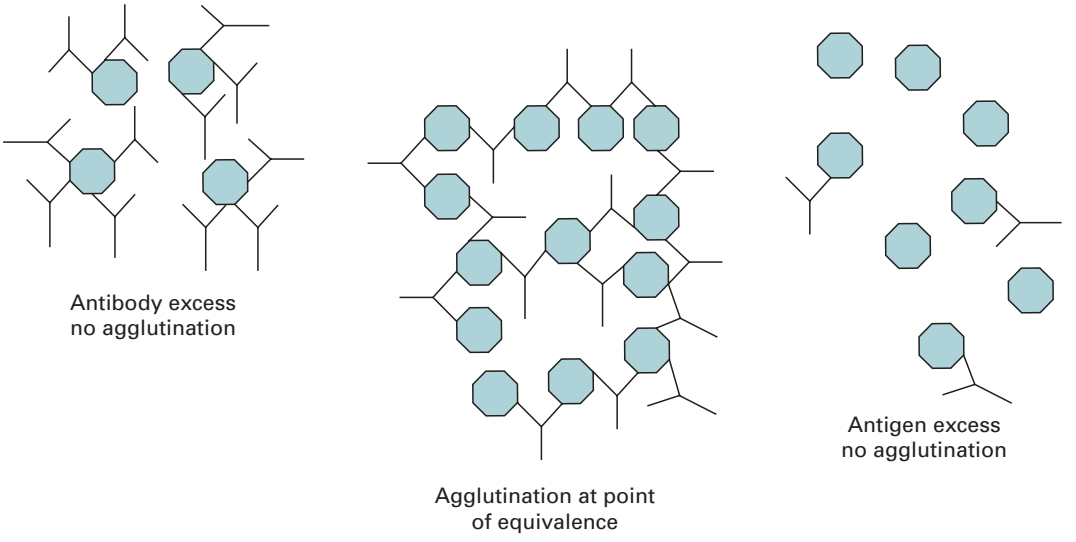

Fig. 7.10 Agglutination reaction.

as an opaque band in an agar plate assay. Agglutination only occurs when there is the right amount of antibody and antigen present. It relies on the fact that as an antibody has two binding sites then each of them can be bound to different antigen particles. As this happens bridges are formed created by the antibody molecules spanning two antigen molecules. The resulting lattice that is created forms a stable structure where antigen and antibody particles are suspended in solution by their attachment to each other. For this to take place there must be a precise amount of antigen and antibody present and this is known as **equivalence**. If too much antibody is present then each antigen molecule will bind multiple antibodies and the meshwork will not develop. If too much antigen is present then each antibody will bind only one antigen particle and no lattice will form. For this reason a dilution series of antibody is often made and a measure of antigen concentration can be made from the end point at which agglutination occurs.

Modifications of the agglutination reaction involve the use of antibody bound to red blood cells or latex particles which allow the reaction to be observed more easily in a liquid phase. Agglutination immunoassays are still used as they provide rapid results with the minimum of equipment. They are commonly used for the detection of viral antigens in blood serum. In commercial tests the antibody concentration in the reagent is provided at a working dilution known to produce a positive for the normal range of antigen concentration.

The Ouchterlony double diffusion agar plate method is the commonest gel-based assay system used (Fig. 7.11). Wells are cut in an agar plate which is then used to load samples and an antibody solution. The antigens and the antibody diffuse through the agar and if the antibody recognises antigen within the gel then a precipitin band is formed. A diffusion gradient is formed through the agar as the reagents progress and so there is no requirement for dilutions to be made to ensure that agglutination occurs.

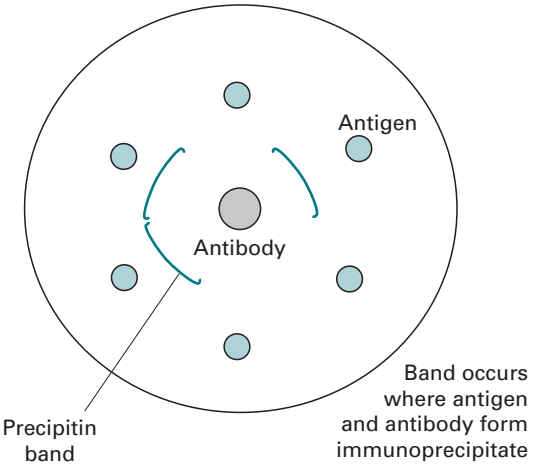

Fig. 7.11 Ouchterlony double diffusion plate.

Polyclonal antibodies can be made to different subspecies of bacteria and these will recognise surface epitopes on them. Because the subspecies are related then they will share some surface epitopes and the closer they are related the more they will share. Antibodies made to one organism will therefore react to a greater or lesser degree to a related organism depending on how many surface epitopes they share.

This has given rise to a systematic method of identification known as **serotyping** which is based on the reaction of microorganisms to antibodies. The system works well with closely related organisms but is not definitive, as it only assesses surface markers. The pattern of precipitin bands obtained to reference antibodies is specific and can be used to assign samples into serotypes. The method was used until very recently to characterise *Salmonella* strains as their pathogenicity can be assessed according to their relatedness to known strains. *Salmonella*s from food and water samples were tested by this method to establish if they had been the cause of food poisoning incidents.

Agglutination reactions using sensitised erythrocytes (red blood cells) or latex particles are carried out in liquid phase usually in small tubes or more recently in round-bottom microtitre plates. As discussed before, the agglutination reaction only occurs at the point of equivalence. A positive agglutination test appears cloudy to the eye as the erythrocytes or latex particles are suspended in solution. A negative result is characterised by a 'button' at the bottom of the reaction vessel which is formed from non-reacted particles. A negative result may be obtained from excess antigen or antibody, as the binding reaction favours the production of small aggregates of antigen/marker particles rather than the agglutination gel.

7.3.1 Enzyme immunosorbent assays

By far, the vast majority of immunoassays carried out fall into the category of enzyme immunosorbent assays. These are routinely used for the diagnosis of infectious agents

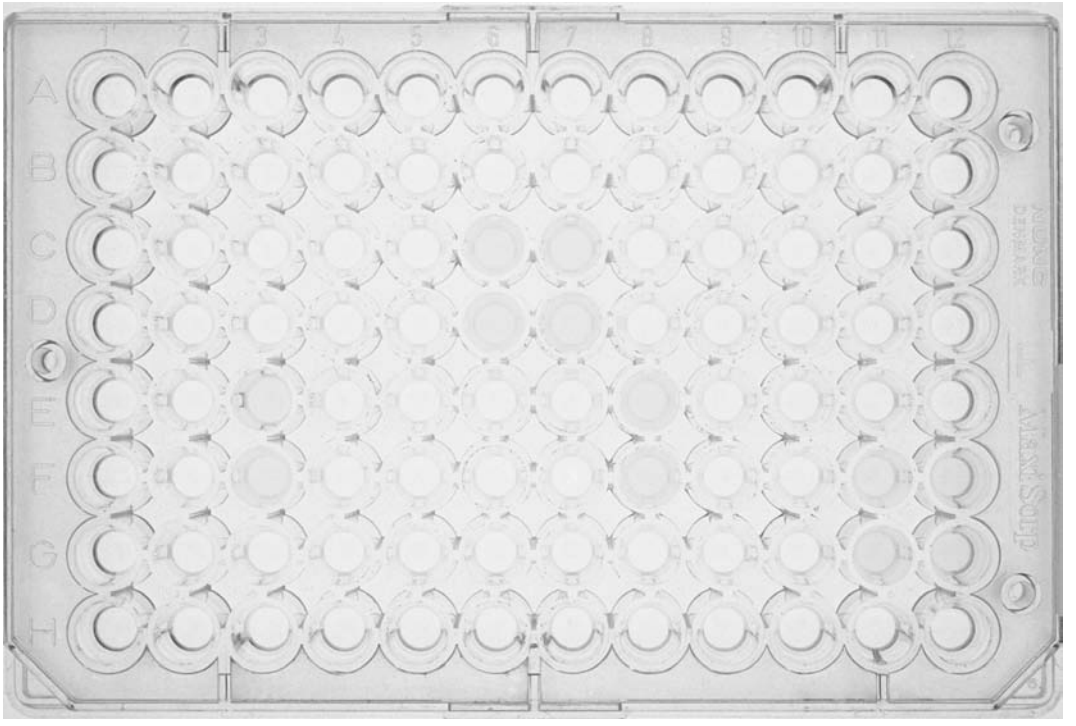

Fig. 7.12 A microtitre plate. (See also colour plate.)

such as viruses, and other substances in blood. The antigen is the substance or agent to be measured. In this technique the antigen is immobilised on to a solid phase, either the reaction vessel or a bead. The most commonly used solid phase is the enzyme linked immunosorbent assay (ELISA) plate. Immobilisation is achieved by the use of a **coating antibody** which actively traps antigen to the solid phase. A second antibody (antibody enzyme conjugate) which is labelled with a **reporter enzyme** is allowed to bind to the immobilised antigen. The **enzyme substrate** is then added to the antigen/antibody/enzyme complex and a reaction, usually involving a colour change, is seen (Fig. 7.12, see also colour section). There are many permutations of this method but all of them rely on the antibody–antigen complex being formed and the presence of it being confirmed by the reactions of the reporter enzyme. These assays rely on a stepwise addition of layers with each one being linked to the one before. The antigen is central to the assay as it provides the bridge between the solid phase and the signal-generating molecule. Without antigen, the antibody enzyme conjugate cannot be bound to the solid phase and no signal can be generated. The coating antibody also concentrates the antigen from the sample as it binds the antigen irreversibly and so the coating layer has the ability to concentrate available antigen until saturation has been reached. This is particularly useful when testing for low levels of antigens in fluids such as blood serum.

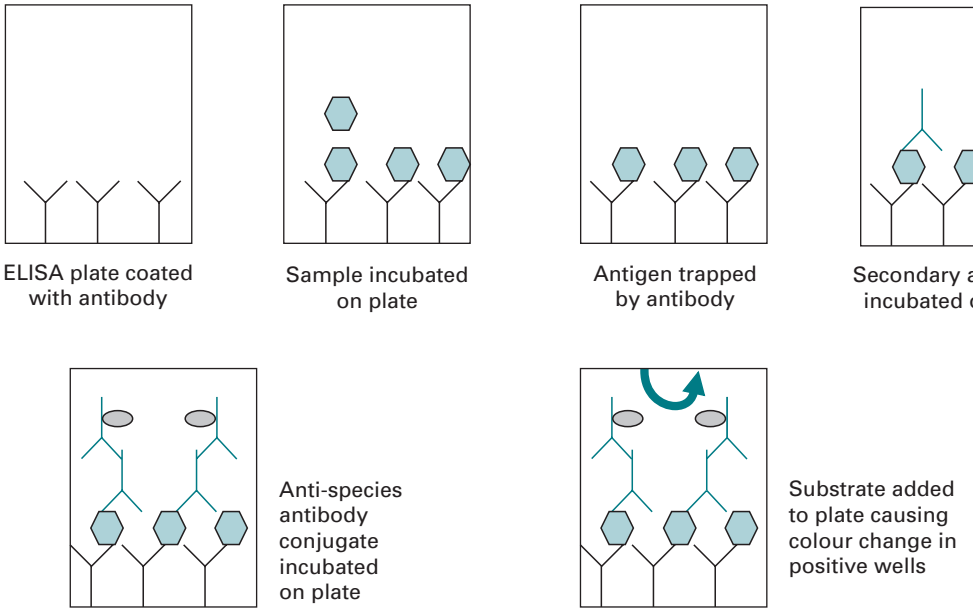

Fig. 7.13 TAS ELISA.

7.3.2 Triple antibody sandwich ELISA (TAS ELISA)

Triple antibody sandwich (TAS) ELISA, also known as indirect ELISA, is a widely used method (Fig. 7.13). It is often used to identify antibodies in patient blood which may be there as the result of infection. As with other immunoassays, layers of reagents are built up, each dependent on the binding of the previous one. The system is used to test patient blood for the presence of hepatitis B virus (HBV) antibodies as a diagnostic test for this disease. In this test HBV coating antibody is bound to the wells of a microtitre plate and HBV coat protein added to them. The live virus is not used as antigen as this would be too dangerous to use in the laboratory. HBV coat protein is made synthetically specifically for use as antigen in this type of test. After incubation and washing, patient serum is added which if it contains antibodies reacts to the antigen. Anti-human antibody conjugated to an enzyme marker is then added which will bind to the patient antibodies. Substrate is then added to identify samples which were positive. The test works well for the diagnosis of HBV infection and is also used to ensure that blood donations given for transfusion are free from this virus.

7.3.3 Double antibody sandwich ELISA (DAS ELISA)

Double antibody sandwich (DAS) ELISA is probably the most widely used immunochemical technique in diagnostics (Fig. 7.14). It is rapid, robust, and reliable and can be performed and the results interpreted with minimal training. The principle is the same as other ELISA techniques in that the antigen is immobilised to a solid phase by a primary antibody and detected with a second antibody which has been labelled with a marker enzyme. The antigen creates a bridge between the two antibodies and the

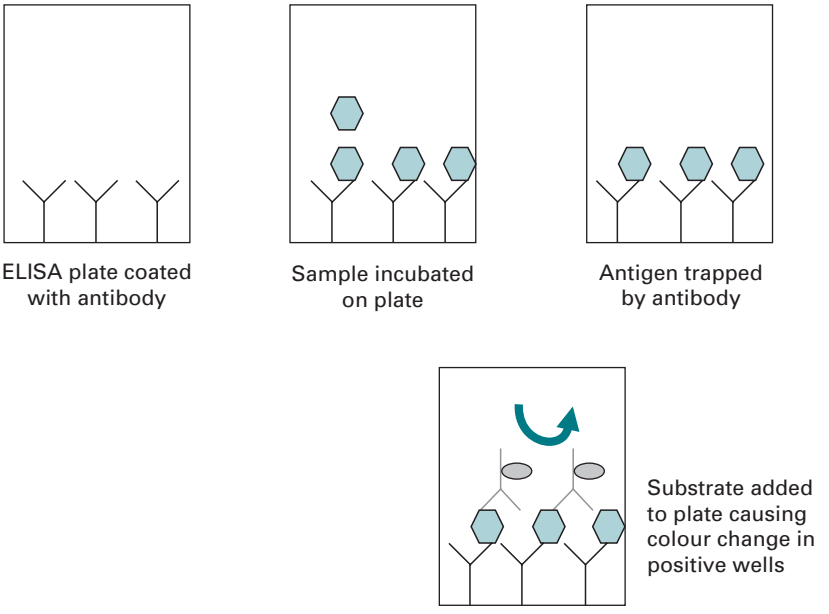

Fig. 7.14 DAS ELISA.

presence of the enzyme causes a colour change in the **chromogenic** (colour-producing) substrate. The marker enzyme used is usually either horseradish peroxidase (HRP) or alkaline phosphatase (AP). Other enzymes have been used and claims have been made for increased sensitivity but this is at the expense of more complex substrates and buffers. In some systems the enzyme is replaced with a radioactive label and this format is known as the **immunoradiometric assay** (IRMA). DAS ELISA is used extensively in horticulture and agriculture to ensure that plant material is free of virus. Potato tubers that are to be used as seed for growing new crops have to be free of potato viruses and screening for this is carried out by DAS ELISA. There are many potato viruses but potato leafroll virus (PLRV) in particular causes considerable problems. PLRV antibodies are coated onto the wells of ELISA plates and then the sap to be tested is added. After incubation, the plates are washed and PLRV antibody conjugated to alkaline phosphatase is added. The plates are incubated and after washing, substrate is used to identify the positive wells. The system again requires the presence of the antigen (PLRV) for the sandwich of antibodies to be built up.

7.3.4 Enhanced ELISA systems

The maximum sensitivity of ELISA is in the picomole range and there have been many attempts to increase the detection threshold for assays beyond this. The physical limitations are based on the dynamics of the double binding event and the subsequent generation of signal above the background substrate value. Most workers have concentrated their efforts on the amplification of signal. Antibody binding cannot be improved as it is primarily a random event modified by the individual avidities of the antibodies themselves. Some improvement in some assays can be

made by temperature modification as some antibodies may perform better at specific temperatures.

There are two basic ways that signal can be amplified in ELISA. More enzyme can be bound by using **multivalent** attachment molecules. Systems using the **avidin–biotin** binding system allow amplification through this route. Both avidin and biotin are **tetravalent** (i.e. they have four binding sites) and it is this property that produces the amplification. The detection antibody is labelled with biotin and the reporter enzyme with avidin. The high affinity and multivalency of the reagents allows larger complexes of enzyme to be linked to the detection antibody, producing an increase in substrate conversion and improved colour development in positive samples. The alternative amplification step is by enhancing the substrate reaction usually by using a double enzyme system. The primary enzyme bound to the antigen catalyses a change in the second enzyme which then generates signal. Both of these methods will increase the signal generated but may also increase the background reaction. Alkaline phosphatase conjugated secondary enzyme can be used to drive a secondary reaction involving NADP dephosphorylation to NAD which is further reduced to NADH by alcohol dehydrogenase. This in turn creates a loop in which a tetrazolium salt is oxidised as the NADH returns to NAD. The tetrazolium salt is chromogenic when in the oxidised state. The cyclic nature of the reaction causes the amplification and increases the observed colour development. Some claims have been made for 'supersubstrates' which work directly with enzyme–antibody conjugates but there is usually little in the way of true gain if standard curves are calculated for the various substrate types.

7.3.5 Competitive ELISA

Competitive ELISA is used in assays for small molecules such as hormones in blood samples where often only a single epitope is present on the antigen (Fig. 7.15). It is quantitative when used in conjunction with a standard curve. The principle is based on competition between the natural antigen (hormone) to be tested for and an enzyme-conjugated form of the antigen which is the detection reagent. The test sample and a defined amount of enzyme-conjugated antigen are mixed together and placed into the coated wells of a microtitre plate. The antigen and conjugated form of it compete for the available spaces on the coating antibody layer. The more natural antigen present the more it will displace (compete out) the conjugated form leading to a reduction in enzyme bound to the plate. The relationship of substrate colour development is therefore inverted; the more natural antigen bound the lower the signal generated. This form of ELISA is routinely used for testing blood samples for thyroxin. Thyroxin is a hormone that is responsible for regulating metabolic rate and deficiencies (hypothyroidism) and excesses (hyperthyroidism) of it will slow or speed up the metabolism. Patients can be given additional thyroxin if required if they are deficient but it is important to establish the baseline level before treating the condition. Competitive ELISA is used for this as an accurate measure of the circulating level of the hormone can be made from a standard curve of known dilutions.

In some assays the enzyme is replaced with a radioactive label and this form of competitive ELISA is known as the radioimmunoassay (RIA).

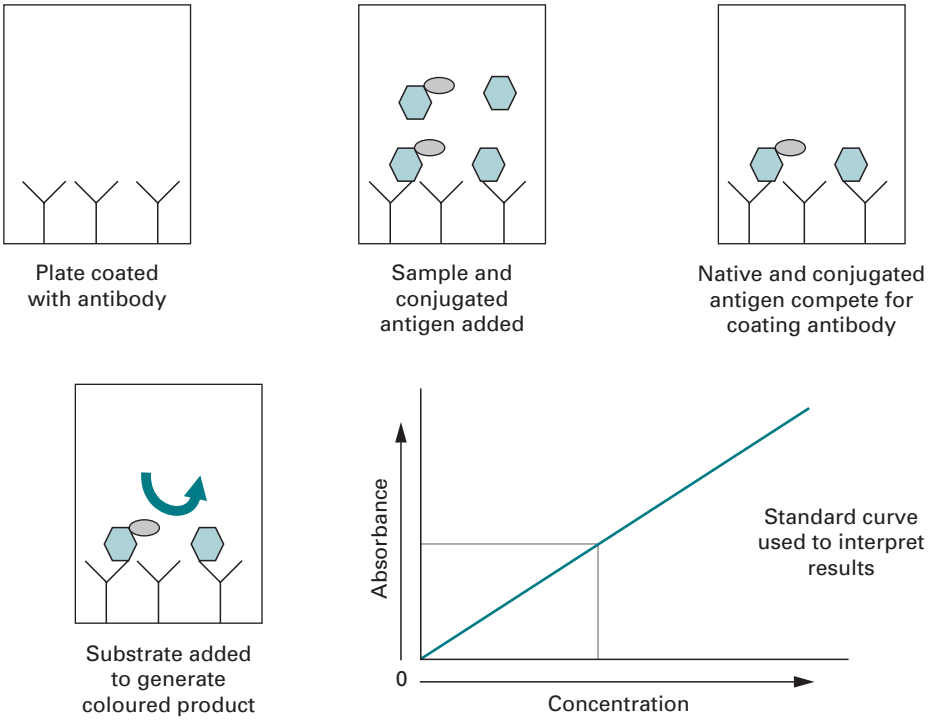

Fig. 7.15 Competitive ELISA.

Competitive ELISA using conjugated antibody can also be used to quantify levels of circulating antibody in test serum. The solid phase has the antigen to which the antibody will attach directly coated on to it. The test serum and the conjugated form of antibody are mixed together and added to the reaction wells. The conjugated and test antibody then compete to bind to the antigen. The level of antibody can again be determined by the reduction in signal observed by addition of the substrate.

7.3.6 Dissociation enhanced lanthanide fluorescence immunoassay (DELFIA)

DELFIA is a time-resolved fluorometric assay which relies on the unique properties of lanthanide chelate antibody labels. The lanthanides will generate a fluorescent signal when stimulated with light of a specific wavelength. The light signal generated has a long decay which enhances the negative to positive ratio of the assay. DELFIA offers a signal enhancement greater than that possible from conventional enzyme-linked assays. The lanthanide chelates are conjugated onto the secondary antibody and as there are a number of lanthanides each with a unique signal which can be used, **multiplexing** (more than one test carried out in the same reaction vessel) is possible. The assay is carried out similarly to standard ELISA and may be competitive or non-competitive. The assay is concluded by adding an enhancement solution which causes dissociation of the lanthanide from the antibody molecules. The signal is generated by stimulating the lanthanide with light of a specific wavelength and measuring the

resulting fluorescence. If europium is used the stimulatory wavelength is 340 nm, and the fluorescence generated is 615 nm.

7.4 IMMUNO MICROSCOPY

7.4.1 Immunofluorescent (IF) microscopy

Immunofluorescent (IF) microscopy uses antibodies conjugated to fluorescent markers to locate specific structures on specimens and allows them to be visualised by illuminating them with ultraviolet light. Fluoroscein and rhodamine are the usual labels used but alternative markers are available. Fluoroscein produces a green fluorescence and rhodamine is red. Microscopes equipped to carry out IF have dual sources of light allowing the operator to view the specimen under white light before illuminating with ultraviolet to look for specific fluorescence. The technique is particularly useful for looking at surface markers on eukaryotic cells but is also used as a whole-cell staining technique in bacteriology.

Membrane studies on whole mammalian cells can be undertaken and the migration, endocytosis (uptake of membrane-bound particles by cells) and fate of labelled receptors studied in real time. Bound receptors in cell membranes frequently migrate to one end of the cell prior to being endocytosed. This phenomenon is known as capping and is easily viewed in living cells using antibodies specific to cell membrane receptors labelled with a fluorescent marker.

7.4.2 Immunosorbent electron microscopy

Immunosorbent electron microscopy (ISEM) is a diagnostic technique used primarily in virology. Virus-specific antibodies conjugated to gold particles are used to visualise virus particles on electron microscopes. The gold is electron-dense and is seen as a dark shadow against the light background of the specimen field. The technique can be used for both transmission or scanning systems. If gold-labelled primary antibodies are not available then anti-IgG–gold conjugated antibodies can be used with the primary antibody in a double antibody system. Both monoclonal and polyclonal antibodies can be used for ISEM depending on the required specificity.

7.5 LATERAL FLOW DEVICES

Lateral flow devices (LFD) are used as rapid diagnostic platforms allowing almost instant results from fluid samples (Fig. 7.16). They are simple to use and contain all of the required components within the strip itself. They are usually supplied as a plastic cassette with a port for applying the sample and an observation window for viewing the result. The technology is based on a solid phase consisting of a nitrocellulose or polycarbonate membrane which has a detection zone which is coated with a trapping

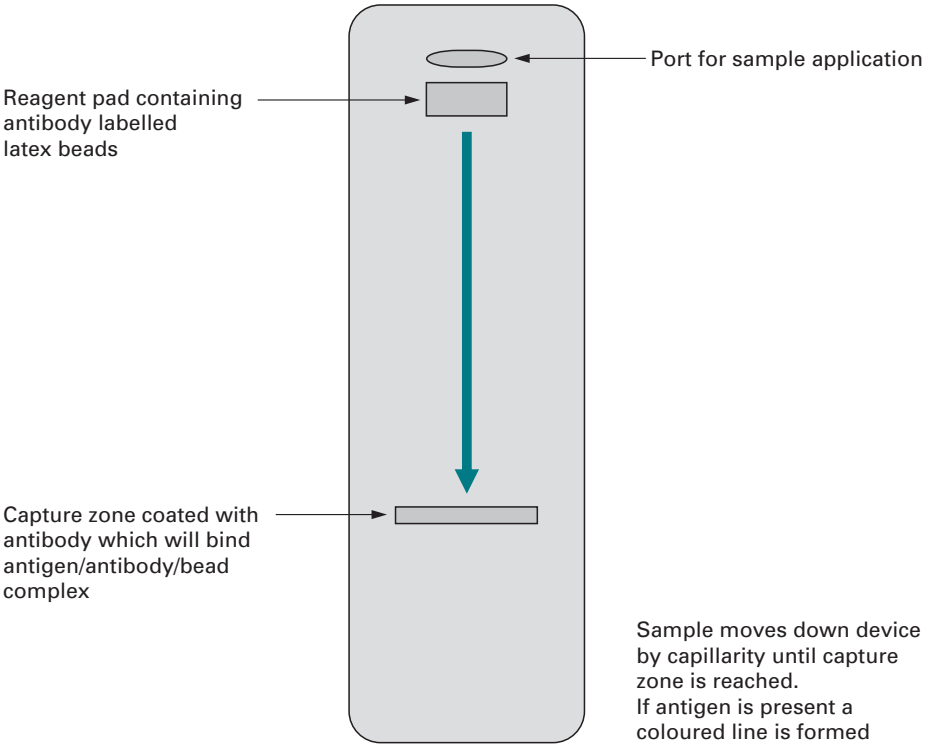

Fig. 7.16 Lateral flow device.

antibody. The detection antibody is conjugated to a solid coloured marker, usually latex or colloidal gold, and is stored in a fibre pad which acts as a reservoir. The solid phase has a layer of transparent plastic overlaying it leaving a very narrow gap which will draw liquid by capillarity. The sample is applied to the reservoir pad through the sample port where it can react with the conjugated antibody if the specific antigen is present. The liquid then leaves the reservoir and travels up the solid phase pad to the location of the trapping antibody. If the sample contains the specific antigen then it will react to both the conjugated and trapping antibody. This results in a coloured line if the sample is positive. If the sample is negative then no coloured line develops. The system lends itself to multiplexing and up to three different antigens can be tested for simultaneously with appropriate trapping antibodies and different coloured marker particles. The technology has been applied to home pregnancy testing and various other 'self-diagnostic' kits. Lateral flow devices are also used by police forces and regulatory authorities for the rapid identification of recreational drugs.

7.6 EPITOPE MAPPING

Epitope mapping is carried out to establish where on the target protein the antibody binds. The method works well with new monoclonal antibodies where it may be necessary to know the precise epitope to which binding occurs. This however can

only be performed where the epitope is linear. A **linear epitope** is formed by amino acids lying adjacent to each other and the antibody binds to the structure that they form. **Non-linear epitopes** are formed from non-adjacent amino acids when they interact with each other in space, as is found in helical or hairpin structures. To carry out epitope mapping the amino acid sequence of the target protein must be known. The sequence is then used to design and make **synthetic peptides** each containing around 15 amino acid residues in length and overlapping with the previous one by about five residues. The synthetic peptides are then coated on to the wells of microtitre plates or onto nitrocellulose membranes and reacted with the antibody of interest. The reaction is visualised by using a secondary antibody enzyme conjugate and substrate. From the reaction to the peptides and the position of the sequence in the native protein it is possible to predict where the epitope lies and also what its sequence is.

7.7 IMMUNOBLOTTING

This technique is also known as **western blotting** and is used to identify proteins from samples after electrophoresis. The sample may be tissue homogenate in origin or an extract of cells or other biological source. The sample may be electrophoresed under **reducing** or **non-reducing** conditions until separation is achieved. This is usually visualised by staining with a general protein stain. The separated proteins are transferred onto a nitrocellulose or polyvinyl membrane either passively or by using an electroblotter. The membrane is treated with a protein-blocking solution to prevent non-specific binding of antibody to the membrane itself. Popular blocking compounds are dried milk or bovine serum albumin. Either direct or indirect antibody systems can be used but often indirect methods are used for reasons of cost. Directly conjugating primary antibodies may be expensive and so very often anti-species enzyme conjugate is used. For indirect labelling the membrane is incubated in antibody solution and after washing, it is treated with a solution of a secondary antibody–enzyme conjugate. Both peroxidase and alkaline phosphatase have substrates that will produce a solid colour reaction on the blot where the antibodies have bound. The substrate reaction can be stopped after optimum colour development, dried and the blots stored for reference. Further details can be found in Section 10.3.8.

The method is particularly useful during development of new antibodies as part of epitope mapping studies.

7.8 FLUORESCENT ACTIVATED CELL SORTING (FACS)

Fluorescent activated cell sorting (FACS) machines are devices that are capable of separating populations of cells into groups of cells with similar characteristics based on antibody binding (Fig. 7.17). The technique is used on live cells and allows recovery and subsequent culture of the cells after separation. Many cell markers are known which identify subsets of cell types and specific antibodies to them are available.

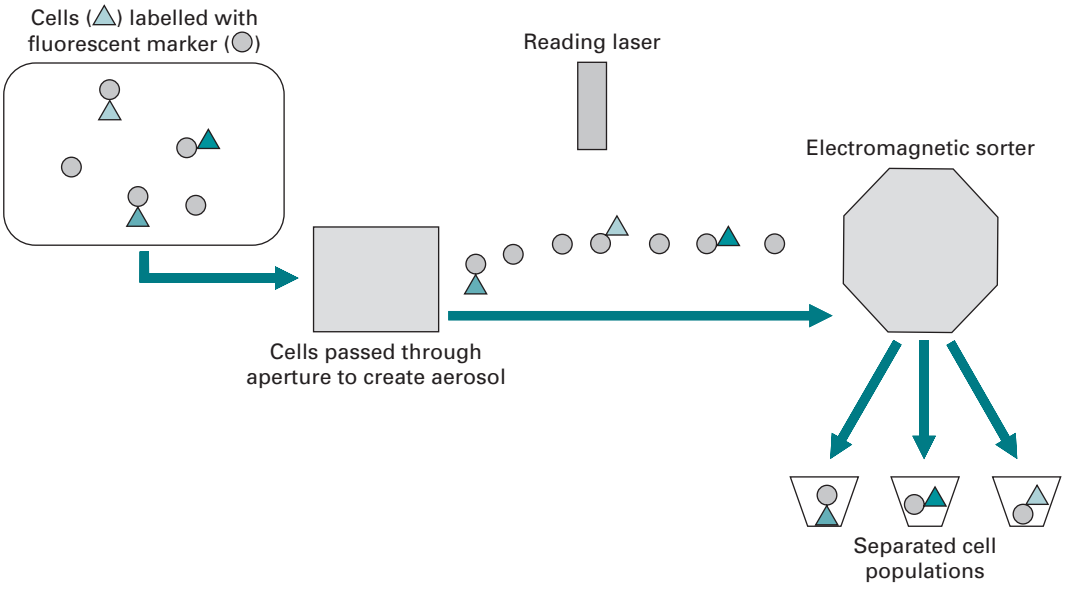

Fig. 7.17 Fluorescent activated cell sorting.

The method can be used quite successfully to separate normal from abnormal cells in bone marrow samples from patients with leukaemia. This can be used as a method of cleaning the marrow prior to autologous (from the patient themselves) marrow transplantation.

The technique is also used for diagnostic tests where the numbers of cell subtypes need to be known. This is of particular use when looking at bloodborne cells such as lymphocytes where the ratios of cell types can be of diagnostic significance. For example, in HIV infection the numbers of specific T cell subtypes are of great diagnostic significance in the progress of the infection to AIDS.

The cells are labelled with the antibodies to specific cell markers labelled with a fluorescent label and then they are passed through a narrow gauge needle to produce an aerosol. The droplet size is adjusted so that each one should contain only one cell. The aerosol is then passed through a scanning Laser which allows detection of the fluorescent label. The droplets have a surface charge and can be deflected by an electron magnet based on their fluorescent label status. The system relies on computer control to effectively sort the cells into labelled and non-labelled populations. The desired cell population can then be recovered and subsequently counted or cultured if desired. More than one label can be used simultaneously so that multiple sorting can be undertaken.

7.9 CELL AND TISSUE STAINING TECHNIQUES

There are many antibodies available that recognise receptors on and structural proteins in cells and tissues and these can be of use diagnostically. Generally immunostaining is carried out on fixed tissues but this is not always the case as it may be important to observe a dynamic event only seen in living cells. Different antibodies

may be required for living and fixed tissues for the same protein, as fixation may destroy the structure of the epitopes in some cases. Fixed tissues are prepared by standard histological methods. The tissue is fixed with a preservative which kills the cells but maintains structure and makes the cell membranes permeable. The sample is embedded in wax or epoxy resin and fine slices are taken using a microtome and they are then mounted onto microscope slides. The antibodies that are used for immunopathology may carry enzyme, fluorescent markers or labels such as gold particles. They may also be unconjugated and in this case would require a secondary antibody conjugate and solid substrate to visualise them. It is important to remember that enzymes such as alkaline phosphatase may be **endogenous** (found naturally) in mammalian tissue samples and their activity is not easily blocked. Often horseradish peroxidase is used as an alternative. Any endogenous peroxidase activity in the sample can be blocked by treating the sample with hydrogen peroxide. Antibodies may recognise structural proteins within the cells and can access them in fixed tissues through the permeabilised membranes. More than one antibody can be used to produce a composite stain with more than one colour of marker being used. Combinations of fluorescent and enzyme staining may also be used but this has to be carried out sequentially. Fluorescent stains can also be used in conjunction with standard histological stains viewed with a microscope equipped with both white and ultraviolet light. Fluorescence will decay in time and although anti-quench products can be used the specimens should not be considered to be permanent. Photographs can be taken of slides through the microscope and kept as a permanent record.

7.10 IMMUNOCAPTURE POLYMERASE CHAIN REACTION (PCR)

Immunocapture PCR is a hybrid method which uses the specificity of antibodies to capture antigen from the sample and the diagnostic power of **PCR** to provide a result. The method is particularly of use in diagnostic virology where the technique allows the capture of virus from test samples and subsequent diagnosis by PCR. It is useful where levels of virus are low such as in water samples and other non-biological sources. The technique can be carried out in standard PCR microtitre plates or in PCR tubes. The antibody is bound passively to the plastic of the plate or tube and the sample incubated afterwards (see Fig. 7.18). After washing to remove excess sample material the PCR reagents can be added and **thermocycling** carried out on the bound viral **nucleic acid**. RNA viruses will require an additional **reverse transcription** step prior to PCR (see also Section 6.8.1).

7.11 IMMUNOAFFINITY CHROMATOGRAPHY (IAC)

Immunoaffinity chromatography can be used for a number of applications. The principle is based on the immobilisation of antibody onto a **matrix**, normally beads, which are then placed into a chromatography column. Antibody may be permanently

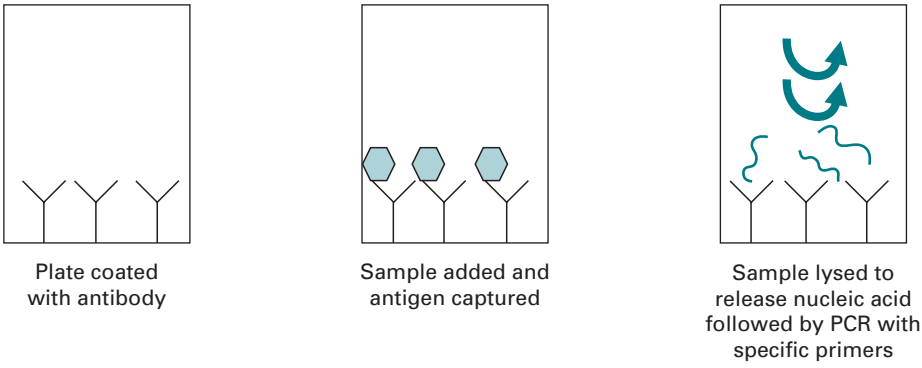

Fig. 7.18 Immunocapture PCR.

linked to the beads by covalent linkage to reactive sites on a resin or bound using protein A or G. Usually, antibody is permanently bound to column beads for most applications as this is a more stable linkage and allows repeated use of the columns following regeneration. IAC may be used as a clean-up method in analytical chemistry to extract small quantities of chemical residues such as **pesticides** from wastewater and other sources. The method also works well for the extraction of biological compounds such as hormones from patient samples. The columns are made by reacting highly purified antibody (monoclonal or polyclonal) with the chromatography beads to form the affinity matrix. Harsh conditions have to be avoided as denaturation of the antibody molecules could occur. A number of proprietary resins are available which have reactive sites suitable for antibody immobilisation. The affinity matrix is loaded into chromatography columns prior to use. Antibody binding of antigen generally occurs best at around pH 7.4 but individual monoclonal antibodies may vary considerably from this pH. Once the sample has been loaded onto the column it should be washed to remove contaminating material from sample fluid. Conditions for elution vary according to individual antibodies and antigens but pH 2.0 buffer, methanol and 10% acetonitrile have all been used successfully. The column can be regenerated after elution by incubating with pH 7.4 buffer. The technique works extremely well for clean-up and concentration of sample from dilute sources prior to additional analysis. Samples eluted from IAC columns may be tested further by high-performance liquid chromatography, ELISA or other analytical techniques.

7.12 ANTIBODY-BASED BIOSENSORS

A **biosensor** is a device that is composed of a biological element and a **physicochemical transduction** part which converts signal reception by the biological entity into an electrical impulse. A number of biosensor devices are available that use enzymes as the biological part of the device. The enzyme is used to catalyse a chemical reaction which generates an electrical charge at an electrode. Antibodies have the potential to

be excellent biological molecules to use for this technology as they can be developed to detect virtually any molecule. The main problem with developing this technology with antibodies has been the lack of adequate physicochemical transduction systems. Three methods have been developed that will provide a signal from antibody binding and these are likely to produce a new generation of biosensors in the future. Antibodies may be bound onto thin layers of gold which in turn are coated onto refractive glass slides. If the slides are illuminated at a precise angle with fixed-wavelength Laser light then electron waves are produced on the surface of the gold. This is known as **surface plasmon resonance** and only occurs if the incident angle and wavelength of light are precisely right. If the antibody binds antigen then the surface plasmon resonance pattern is changed and a measurable change in emitted energy is observed.

Fibre optic sensors have also been developed which rely on the natural ability of biological materials to fluoresce with light at defined frequency. The reaction vessel is coated with antibody and the fibre optic sensor used to illuminate and read light scatter from the vessel. The sample is then applied and the sample vessel washed. The fibre optic sensor is again used to illuminate and read backscatter from the vessel. Changes in the fluorescence will give a change in the observed returned light.

A third approach relies on changes in crystals as a result of surface molecules bound to them. **Piezoelectric crystals** generate a characteristic signature resonance when stimulated with an alternating current. The crystals are elastic and changes to their surface will produce a change in the signature resonance. The binding of antigen to antibody located on the surface of the crystal can be sufficient to alter the signature and therefore induce a signal indicating that antigen has been detected by antibody.

7.13 THERAPEUTIC ANTIBODIES

Therapeutic antibodies fall into a number of different classes but are all designed to bind to specific structures or molecules to alter cellular or systemic responses *in vivo*. The simplest of these are the **inhibitory** systemic (found throughout the body) antibodies that will bind to substances to render them ineffective. At their crudest, they consist of hyperimmune serum and are used to alleviate the symptoms of bites and stings from a number of poisonous animals. **Antivenom** produced in horses for treatment of snake bite is a good example of this. Hyperimmune serum derived from human patients who have had the disease has also been used prophylactically (reduce the risk of disease) after exposure to pathogenic viruses. Hyperimmune serum is available to help to treat a number of pathogenic viral conditions such as West Nile Fever, AIDS and hepatitis B. These are used after exposure to the pathogen, for example by needle-stick injury, and help to reduce the risk of infection occurring. The next class of therapeutic antibodies are those that bind bioactive molecules and reduce their effects *in vivo*. They are all monoclonal and have a number of targets which help to alleviate the symptoms of a number of human diseases. One of the major targets for this approach are systemic cytokines which have been implicated in

the progression of diseases such as arthritis; results using antibody therapy have been encouraging. Monoclonal antibodies can also be used to reduce the numbers of specific cell types *in vivo* by binding to surface markers on them. The binding of the antibody to the cells alerts the immune system and causes the cells to be cleared from circulation. Chimeric (formed from two sources) mouse/human monoclonal antibodies consisting of mouse variable regions and human constant regions which are specific to the B cell marker **CD20** have been used successfully for the treatment of systemic lupus erythematosus. This disease is characterised by the development of aberrant B cells secreting autoantibodies which cause a number of immune phenomena. The decrease in circulating B cells reduces the number producing the autoantibodies and alleviates some of the symptoms.

Agonistic (causing upregulation of a biological system) monoclonal antibodies are therapeutic antibodies which have the ability to influence living cells *in vivo*. They upregulate cellular systems by binding to **surface receptor molecules**. Normally, cell receptors are stimulated briefly by their ligand (substance that binds to them) and the resulting upregulation is also brief. Agonistic monoclonal antibodies bind to the receptor molecule and mimic their ligand, but have the capacity to remain in place for much longer than the natural molecule. This is due to the fact that the cell finds it much more difficult to clear the antibody than it would the natural ligand. The action of agonistic antibodies is incredibly powerful as the internal system cascades that can be generated are potentially catastrophic for both the cell and the organism. Their use has been mainly restricted to induction of **apoptosis** (programmed cell death) in cancer cells and only where a known unique cellular receptor is being stimulated.

There are a number of therapeutic inhibitory antibodies available and all of them downregulate cellular systems by blocking the binding of antigen to receptor. They behave as **competitive analogues** to the inhibitor and have a long **dwell time** (the time they remain bound) on the receptor increasing their potency. They may block the binding of hormones, cytokines and other cellular messengers. They have been used successfully for the management of some hormone-dependent tumours such as breast cancer and also for the downregulation of the immune system to help prevent rejection after organ transplantation.

These therapeutic antibody types need to be carefully engineered to make them effective as treatment agents. The avidity and affinity of the antibodies is critical to their therapeutic efficacy as their specific binding ability is critical to their length and specificity of action. Additionally, they must not appear as 'foreign' to the immune system or they will be rapidly cleared by the body. Often, the original monoclonal antibody will have been derived using a mouse system and as a result is a murine antibody. These antibodies can be humanised by engineering the cells, retaining the murine binding site and replacing the constant region genes with human ones. The resulting antibody escapes immune surveillance but retain their effective binding capacity. Natural antibodies may remain in the circulation for up to 6 weeks but engineered antibodies survive a much shorter time. The shortened survival time is due to the **humanisation** which still leaves a degree of murine antibody visible to the immune system. Each engineered, therapeutic antibody has a different half-life *in vivo* and this factor is of great importance when baseline dosage is being established. All of

the currently used therapeutic antibodies may cause side effects in patients and so this line of therapy has only been exploited where the benefits outweigh the problems that may be encountered. Great success has been seen in the treatment of prostate cancer in men and breast cancer in women using humanised monoclonal antibodies which bind to hormone receptors on the tumour cells and inhibit their growth as a result.

7.14 THE FUTURE USES OF ANTIBODY TECHNOLOGY

Antibodies are incredibly useful molecules which can be designed to detect an almost limitless number of antigens. They are adaptable and will operate in many conditions. They can be used in both diagnostic and therapeutic scenarios. In the future there will be a rise in the availability of therapeutic antibodies both for the up- and downregulation of cellular and systemic responses. Cancer therapy and **immune modulation** of autoimmune phenomena are probably the two areas where greatest developments will take place.

Biosensors for the detection of disease will become increasingly available as will multiple **lab on a chip** (LOC) formats. LOC devices are miniaturised devices that are capable of handling microscopic amounts of liquids and perform a number of laboratory assays in miniature. They are frequently fully automated and can give rapid results without the equipment normally required for laboratory assays. They have the added advantage that they can be used in field situations as they are becoming increasingly portable.

The use of non-animal systems for antibody generation will be exploited more fully with more use of phage display and other DNA library based systems.

7.15 SUGGESTIONS FOR FURTHER READING

Burns, R. (ed.) (2005). *Immunochemical Protocols*, 3rd edn. Totowa, NJ: Humana Press.

Coligan, J. (2005). *Short Protocols in Immunology*. New York: John Wiley. (A good background book which gives detail of immunological protocols and how they can be used to investigate the immune system.)

Cruse, J. and Lewis, R. (2002). *Illustrated Dictionary of Immunology*, 2nd edn. Boca Raton, FL: CRC Press. (An excellent book which describes in detail immunological processes and how they interact. A good balance of text and graphics.)

Howard, G. and Kaser, M. (2007). *Making and Using Antibodies: A Practical Handbook*. Boca Raton, FL: CRC Press. (An excellent book which describes in detail methods for producing, validating, purifying, modifying and storing antibodies.)

Subramanian, G. (ed.) (2004). *Antibodies,* Volume 1, *Production and Purification*. Dordrecht: Kluwer Academic. (This book gives good coverage of methods for antibody production, purification, modification and storage.)

Wild, D. (2005). *Immunoassay Handbook*, 3rd edn. New York: Elsevier. (This book describes in detail background to many clinical immunoassays and how to design and validate them.)