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SECTION 3

Antigen–antibody reactions

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Introduction

Although there are many different types of antigen–antibody reactions, blood bankers are often concerned with reactions between antigens on red blood cells and antibodies in serum/plasma. These antigen–antibody reactions can occur observably in varying proportions, with regard to volumes and strength of reactants used. They are also reversible and are influenced by many factors. Antigen–antibody reactions are enhanced in the laboratory to make them observable, in an effort to draw practical conclusions and report on clinical conditions with accuracy. Provided that the correct methods are followed, it is usually not difficult to determine blood groups and the nature and specificity of antibodies. Occasionally, however, this is more challenging and it sometimes takes considerable knowledge, skill and experience to accurately assess anomalous test results, determine what should be performed next, carry out further tests appropriately and then correctly interpret the results.

This section should not to be confused with *Section 4: Principles of laboratory techniques*, in which the principles and outlines of methodologies are given. That section draws on the concepts that explain reactions. It should also not to be confused with the causes of false results. False results occur when something goes wrong within the testing environment – either because of human error or the failure of materials or equipment used or some other factor. The causes of false results are described in detail in *Section 4: Principles of laboratory techniques*.

Learning objectives

By the end of this section, the student should be able to describe the common types of antigen–antibody reactions, discuss the factors that influence them and use this knowledge to carry out laboratory tests in such a way as to obtain the best results:

- First and second stages of antigen–antibody reactions
- Common types of antigen–antibody reactions
  - haemagglutination
  - sensitization
  - haemolysis
  - neutralization
  - precipitation
- Factors that influence antigen–antibody reactions
- Use of proteolytic enzymes
- Use of high molecular mass substances
- Use of low ionic strength saline solution
- Use of labelled antibodies
- Role of complement
  - factors that influence the action of complement in laboratory tests
- Role of antihuman globulin
  - application of antiglobulin tests in the laboratory
- Polyclonal and monoclonal antibodies.

First and second stages of antigen–antibody reactions

When a blood sample is drawn, it is taken either into a dry test tube or into a test tube containing an anticoagulant. Serum is the fluid part of a blood specimen taken into a dry test tube, and allowed to clot. It has similar properties to plasma, but the coagulation factors are missing because they have been utilized in the clotting process. The blood sample in the plain tube coagulates and in a short time the clot will retract (shrink) sufficiently for the serum to be clearly visible. To prevent coagulation, the blood sample may be taken into an anticoagulant, such as acid citrate dextrose (ACD) or ethylenediaminetetraacetic acid (EDTA).

Laboratory tests involving reactions between serum/plasma containing blood group antibodies, and red cells expressing the corresponding antigens, take place in two stages. Although the stages are distinct, they need not necessarily be entirely separate entities; the two stages may overlap to some extent.

First stage

This involves antigens and antibodies randomly bumping into each other in the test environment, and when this occurs at the antigen site, the actual attachment of antibody to antigen takes place. It usually happens very quickly and is affected by many variables. The reaction is not visible.

Second stage

This involves the demonstrable effect of attachment of antibody to antigen. This stage takes a longer time to develop and may need to be enhanced in the laboratory in order for it to become observable.

The most widely used strategy in the laboratory to enhance the visibility of antigen–antibody reactions is centrifugation.

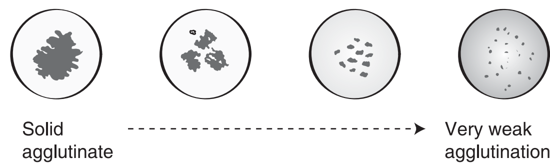

Fig. 3·1 Range of reaction strength – haemagglutination.

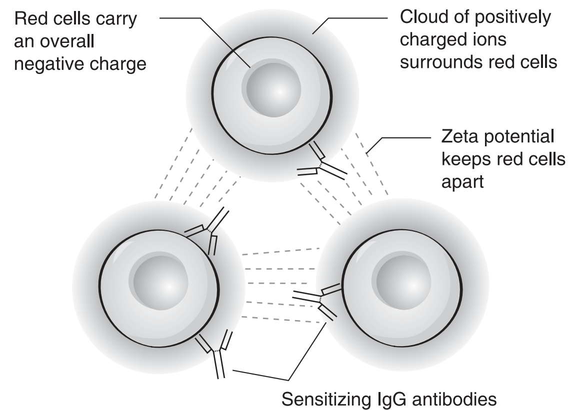

Fig. 3·2 Red cell sensitization showing ionic cloud.

After allowing sufficient time for antibody to recognize and react with antigen, which may be within seconds or may take much longer, up to 1 hour, tests can be centrifuged to force the cells closer together. In this way agglutination may be enhanced, whereas cells that have not reacted with antibody remain unagglutinated.

Common types of antigen–antibody reactions

Antigen–antibody reactions that are of particular relevance to blood bankers are introduced below. This is followed by the factors that influence reactions, and the various agents that may be used to improve or enhance them.

Haemagglutination

Because they have similar negative electrical charges, red cells are kept apart. This is the natural repelling force that exists between molecules with similar electrical charges, and is called zeta potential. The negatively charged red cells in saline or suspending medium attract a cloud of positively charged ions around them. IgM antibodies, being 300 Å long, are able to span the distance between adjacent red cells and as a result, bring about haemagglutination of cells with the corresponding antigen. This is what typically happens in ABO blood grouping tests.

The agglutination of red cells in this way is correctly called haemagglutination, although it is often simply referred to as agglutination. It takes place when serum/plasma antibody (e.g. anti-A) is mixed with red cells carrying the corresponding antigen (i.e. A antigen). This reaction may occur in a test tube, or on a microscope slide, or in a microwell. The result is the development of a three-dimensional latticework of red cells held together by antibodies and visible as clumping. Figure 3·1 shows a range of haemagglutination reaction strengths, from strong agglutination that is easily visible, to very weak agglutination that is difficult to discern.

Sensitization

Sensitizing antibodies are IgG antibodies that are about 120 Å in length. Although they are able to sensitize red cells with the corresponding antigens, zeta potential must be reduced or altered for the smaller IgG antibodies to achieve haemagglutination. Additives such as bovine serum albumin or proteolytic enzymes like papain or bromelin are able to reduce zeta potential, and thereby change the environment surrounding the red cells so as to allow sensitized cells to become agglutinated.

The sensitization reaction, although just as significant as haemagglutination, is not observable. Figure 3·2 illustrates red cell sensitization and the ionic cloud of positively charged ions surrounding the negatively charged red cells, kept apart by zeta potential.

Laboratory tests have therefore to be modified in a prescribed way to enable sensitization to become observable *in vitro*. There is a notable exception to this rule, and that is agglutinating IgG anti-A and anti-B, mentioned in *Section 4: Principles of laboratory techniques*.

There are two commonly used ways to cause sensitized cells to become agglutinated, and these are described in detail later in this section. In brief, the principles of these methods are:

- Reduction of zeta potential – substances such as proteolytic enzymes or bovine serum albumin may be used to reduce the repelling force between red cells, bringing them closer together so that if sensitized, they are able to become agglutinated.
- Bridging the gap between sensitized cells – antihuman globulin (AHG) is an antibody to human globulin, and is used in the laboratory to react with sensitizing globulins (antibodies), bridging the gap and bringing about the agglutination of the cells they had sensitized.

Antibody affinity and avidity

- The strength of the actual bond between a single antibody combining site and a single epitope is known as the affinity of the antibody, and relates to its goodness of fit with the corresponding antigen.

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- The combined strength of multivalent antibody binding to many epitopes on the same carrier (such as a red blood cell) is known as the avidity of the antibody. In blood banking terms, this condition could apply to IgM or IgG antibodies, as both have more than one binding site per molecule.

Prozoning

On rare occasions, an undiluted antibody with high avidity, when mixed with a suspension of red cells containing the corresponding antigen, will fail to show any demonstrable reaction *in vitro*, but will do so when diluted and mixed with these same cells. This is the result of antibody excess in the neat serum/plasma that prevents the development of a regular latticework of visible agglutination. When suitably diluted, the number of antibody molecules is reduced sufficiently to allow normal agglutination of the cells to take place. This is discussed in more detail under the causes of false results in *Section 4: Principles of laboratory techniques.*

Qualitative and quantitative agglutination tests

- Qualitative tests determine the presence or absence of an antigen or antibody. Blood grouping tests that are performed to determine the presence or absence of an antigen using an antibody of known specificity are qualitative tests. For example, ABO blood grouping may be carried out by cell (or forward) grouping using reagent anti-A and anti-B with red cells of unknown group, and reverse grouping of unknown serum/plasma with reagent A and B cells.
- Quantitative tests determine the highest dilution at which an antibody is able to react with its corresponding antigen. This endpoint is the titre of the antibody, expressed as a reciprocal (or inverse) of the highest dilution at which agglutination was observed. For example, if the highest dilution at which a reaction is observable is 1 in 32, then the titre is expressed as 32. Quantitative tests may also be used to determine the international units of antibody in a serum/plasma sample.

Haemolysis

Sometimes antigen–antibody reactions result in lysis, which is the breakdown or rupture of the cell membrane on which the epitopes or antigenic determinants are situated. When this involves red blood cells, it is called haemolysis, and causes the release of haemoglobin. This is an observable reaction in laboratory tests and must be noticed and recorded. For this to occur, the antibody involved in the reaction must be able to utilize complement, present in fresh serum or in the bloodstream. Complement is a group of proteins that when triggered by antibody adherence (attachment or sensitization) to the cell, act in a chain reaction to attack and break or rupture the cell membrane. Haemolysis of this nature is therefore a demonstrable endpoint of certain antigen–antibody reactions. Figure 3·3 illustrates the appearance of haemagglutination and haemolysis in test tubes, compared with an unagglutinated test.

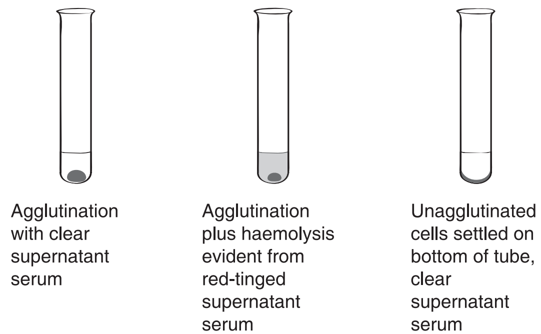

Fig. 3·3 Haemagglutination and haemolysis in test tubes, and an unagglutinated test.

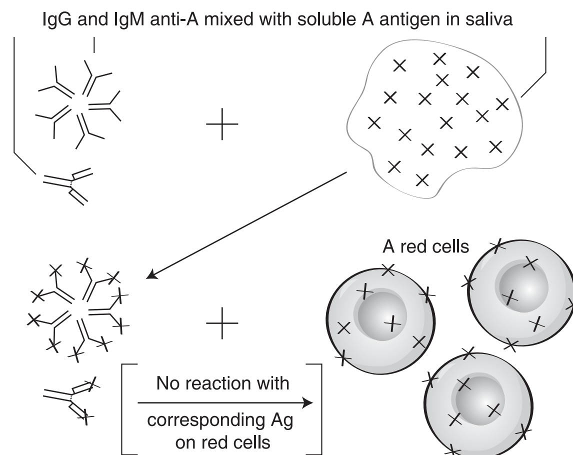

Fig. 3·4 Neutralization of anti-A by soluble A antigen.

Neutralization (agglutination inhibition)

The majority of individuals secrete water soluble ABO blood group antigens in their body fluids such as saliva. Should saliva containing A antigens be mixed with anti-A in the laboratory, then this anti-A will become neutralized. It will therefore be unable to react as expected with group A red cells subsequently added to the mixture. When an antibody has been neutralized, it means that its reactive sites have been blocked by antigen, which is free (not attached to a carrier molecule like a red blood cell). Such a neutralization reaction is usually not observable and can only be deduced by the subsequent lack of agglutination when in contact with the corresponding antigen. Figure 3·4 is a diagrammatic

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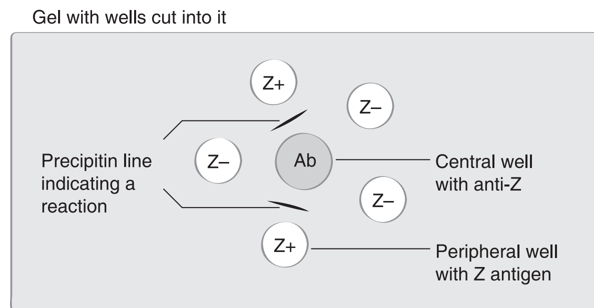

Fig. 3·5 Precipitation by immunodiffusion.

representation of the process of neutralization of anti-A by soluble A antigen so that it can no longer agglutinate red cells containing A antigen.

Precipitation

Precipitation is commonly seen as a precipitin line, such as in immunodiffusion, when antibody and antigen are added to different wells cut into gel set onto a microscope slide. For example, anti-Z may be added to a central well cut into the gel, and several unknown samples that may carry the corresponding soluble Z antigen added to different wells surrounding it. After allowing time under the correct conditions, for diffusion of all the solutions into the gel, it is examined for precipitin lines. If the antibody diffusing out from the central well comes into contact with the corresponding antigen that has diffused out from one of the peripheral wells, then a white line of reaction may be seen in the gel between them – where they have come into contact with each other. This is formed by the precipitation of insoluble antigen–antibody complexes. Precipitin lines in an immunodiffusion gel are shown in Figure 3·5.

Factors that influence antigen–antibody reactions

Distance between reactive sites on antibodies

IgM antibody molecules are 300 Å long and able to react observably by haemagglutination of red cells in saline. IgG antibodies are 120 Å long and usually sensitize cells in saline.

Electric repulsion between red cells – zeta potential

The repelling force between red cells that carry the same negative electrical charge is called zeta potential, which prevents the agglutination of sensitized red cells in saline. Zeta potential must therefore be reduced or altered in some way for the smaller IgG antibodies to be able to achieve agglutination.

Site of the antigenic determinants

It is thought that some antigens (such as the A and B antigens) protrude from the red cell surface farther than others (such as the Rh antigens). Because of this, the actual distance between antigens on adjacent cells may vary to a certain extent, so affecting the nature of the reaction or the ability of corresponding antibodies to react with them.

Number of antigenic determinants

It is easier for antibodies to react with antigens, which are in abundance on each red cell, than to react with antigens that are located only sparsely on the cells. Cells that are homozygous for a particular antigen may carry more antigen sites (antigenic determinants or epitopes) than cells which are heterozygous. This is termed the dosage effect. For example, S positive red cells that are genetically *S/S* (with a double dose of *S*) may react more strongly with anti-S than cells which are heterozygous *S/s* (with a single dose of *S*), depending on the anti-S used in the tests.

Goodness of fit

Antigens and antibodies react in a 'lock-and-key' way. When the combination between lock and key is precise, then the goodness of fit is high, and the reaction will be stronger; a weak fit results in a weaker reaction. The degree of goodness of fit is also known as antibody affinity.

Effects of time

Reactants should be incubated for the optimum time for a good antigen–antibody reaction to develop. Too short an incubation period means that the antigen and the antibody may not have had sufficient time to form a good reaction. On the other hand, prolonged incubation may cause antigen–antibody complexes to dissociate. The best balance should be determined, documented and followed each time tests are performed.

Effects of temperature

Cold antibodies react well at +2°C to +10°C, agglutinating or sensitizing red cells in the cold. These antibodies will usually dissociate from the cells when the temperature of the tests is raised. Thus, cold antibodies may be eluted from red cells by raising the temperature from +2°C to +37°C.

Most IgG antibodies react best with the corresponding antigens at +37°C. At this temperature, the speed of their reaction is also increased. In order to dissociate antigen–antibody complexes formed by antibodies with an optimum reaction temperature of +37°C, one would have to raise the

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temperature to about +56°C. At this temperature, antibody would be eluted (removed or forced to be released) from the cells and could then be isolated and further tested. Red cells however, become denatured at temperatures in excess of +50°C and would have to be discarded.

Effects of pH

pH is the measure of alkalinity or acidity of a solution. The optimal pH range for red cell antigen–antibody reactions to occur is between of 6·5 and 7·0, with an acceptable range of pH of 6·0–8·0. Outside this range, results become unreliable.

Effects of ionic strength

Negatively charged red blood cells attract a 'cloud' of positive ions from the surrounding medium, which is usually saline – sodium chloride dissolved in water. What is commonly known as normal ionic strength saline solution is isotonic with blood; it has the same tonicity as blood. It is a solution of about 0·85% to 0·9% weight to volume of sodium chloride in water. Low ionic strength saline solutions are commonly used to increase the sensitivity of antigen–antibody reactions, and details on this are to be found later in this section.

Concentration of antigen and antibody

Although most antigen–antibody reactions provide observable results at various concentrations of either antigen or antibody, the best results are obtained when a large number of antibody molecules are bound to each cell.

Number of fragment antigen binding sites

IgM antibodies have between 5 and 10 fragment antigen binding (Fab) sites, whereas IgG antibodies are monomers with a maximum of 2 Fab sites. To bring about the agglutination of two adjacent red cells, an IgM antibody could bind with several antigens on one cell and several on the second cell and form a fairly strong bond. An IgG molecule, though, could bind to only one antigen on one cell and one antigen on another cell, and unless it is an avid antibody, may form a weaker bond. In both cases, many molecules of antibody are required to result in a demonstrable reaction, but the principle remains the same.

Use of proteolytic enzymes

The way in which proteolytic enzymes (proteases) enhance antigen–antibody reactions is not clearly understood. It is thought that they enhance reactions because they cause the sialic acid residues around the red cells to be reduced and therefore reduce the zeta potential or repelling force between them. By the reduction in zeta potential, cells are able to approach each other more closely. Cells that were sensitized by the shorter IgG antibody molecules are therefore able to become agglutinated, showing that a reaction took place initially.

The addition of enzyme to unsensitized cells has no visible effect. It is important to take into consideration, however, that enzymes are able to modify or alter certain red cell antigen expression, so that the corresponding antibodies are not able to recognize these antigens. As a result of this, certain antibodies will not usually be demonstrable using enzyme methods, and thus enzyme methods should supplement but not replace other techniques. Antigens that are not recognizable after the addition of enzyme include M, N, S, Fy^a and Fy^b, and to a certain extent, K. On the other hand, some antigen–antibody reactions are enhanced by the addition of enzyme, and this includes antibody reactions to the Le^a, Le^b, I, P_1 and Rh antigens. More information is to be found in *Section 6: Blood group systems.*

There are several methods for using proteolytic enzymes or proteases in blood grouping work.

One-stage technique

One-stage methods are simple and fast. One volume each of serum/plasma, cells and enzyme are added in quick succession and in this order. Modification of the cells takes place during incubation.

Two-stage technique

This is the more sensitive method, but is more labourious. Red cells are pretreated with enzyme and then washed in saline before being mixed with the serum/plasma. One part serum/plasma is then mixed with one part treated cells and incubated. There is no dilution of the serum/plasma under test by the addition of enzyme separately, as for the one-stage method.

Figure 3·6 illustrates the action of enzymes on sensitized red cells, and how agglutination is achieved by the reduction of zeta potential.

The choice of enzyme depends largely on the preferences within the laboratory, although papain is most commonly used. Enzymes may be obtained from the following sources:

- Pineapple stem: source of bromelin
- Dried latex of fig tree: source of ficin
- Latex of papaya fruit: source of papain
- Extract of pig stomach: source of trypsin.

Raw or concentrated enzymes are potent chemicals and care should be taken when working with them. For example, powdered ficin may cause a skin rash and may lead to conjunctivitis if accidentally rubbed into the eyes. The enzymes listed, as well as pretreated red cells, are available commercially.

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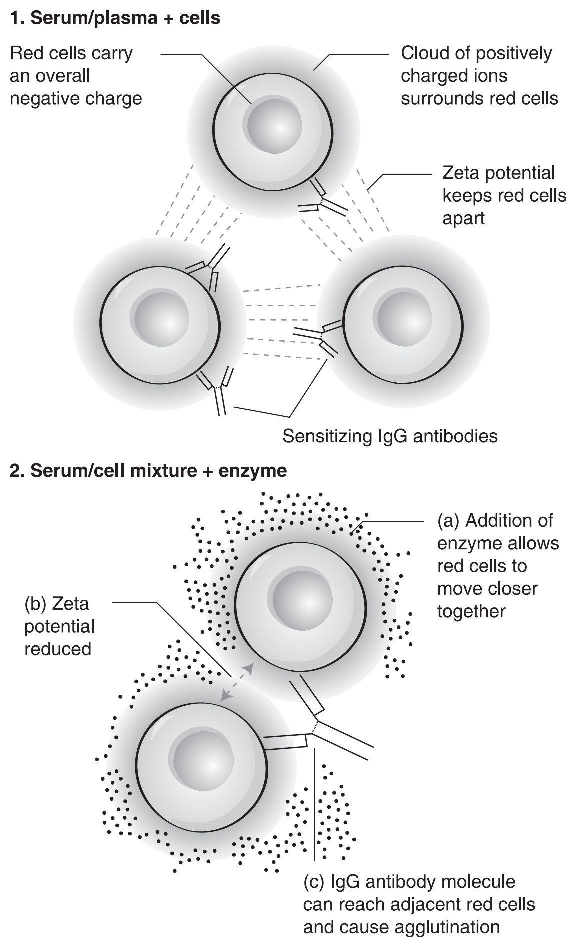

Fig. 3·6 Action of enzymes on sensitized red cells.

Use of high molecular mass substances

There is a range of substances of high molecular mass that can enhance (potentiate) or demonstrate that a reaction has occurred between cells and sensitizing antibodies. These include the following:

- Bovine serum albumin
- Polyethylene glycol
- Polybrene (a polymer of hexadimethrine bromide)
- Polyvinylpyrrolidone
- Gelatin
- Gum acacia.

Note: Potentiators may also be added to reagents to enhance their reactivity.

The addition of 22% bovine serum albumin, particularly in low ionic strength saline (LISS), has been found to increase the sensitivity of the tests, but this may be in part due to the low ionic strength medium, in which it is diluted. Albumin also enhances reactions when used in a normal ionic strength saline (NISS), which was routinely used before LISS became available. One theory is that albumin increases the dielectric constant, a measure that relates to stored electrical energy. Being a dipolar molecule, both positively and negatively charged, albumin is forced to rotate in the serum/plasma–cell mixture, as it is alternatively attracted and repulsed by the negatively charged red cells. In this way zeta potential is reduced and as a result the cells are able to move closer together, so that should these cells already be sensitized, they are then able to become agglutinated.

Polyethylene glycol (PEG) in a low ionic strength environment is one of the best potentiators to increase antibody uptake onto cells with the corresponding antigen. It increases the sensitivity of tests involving clinically significant antibodies and decreases the interference of clinically insignificant antibodies. It may be used in both manual and automated systems, but care should be taken when centrifuging, as red cells clump tightly and may not be dispersed. Antiglobulin tests in which the serum/plasma-cells-PEG mixture is washed, should not present this problem.

Polybrene is an additive of high molecular mass that is introduced to the serum/plasma–cell mixture after the incubation period. Although it is capable of detecting both ABO and IgG incompatibility in crossmatch tests, it may fail to clinically detect significant antibodies within the Kell blood group system. It is also prone to false positives with cells coated with complement.

Use of low ionic strength saline solution

Low ionic strength saline solution is commonly used for red cell suspensions, and is commercially available. It is important that the manufacturer's instructions are carefully followed, otherwise false results may occur. It has two major impacts:

- Reduces the incubation time required for tests
- Increases the amount of antibody uptake onto red cells expressing the antigen.

Low ionic strength saline solution should not be used when the quantity of serum/plasma is changed in the test, either by adding excess serum/plasma, or when serum/plasma is diluted in saline, such as for titration studies, where the proportion of saline diluent to serum/plasma antibody increases from test tube to test tube. It is important that the ratio (and strength) of cell suspension to the volume of serum/plasma remains within the limits stipulated by the manufacturer of the LISS. Failure to ensure that tests are carried out according to these concentrations could lead to false results.

Use of labelled antibodies

Another way to demonstrate antigen–antibody reactions is to put a label or tag onto a known antibody, and then use this tag or label to give demonstrable evidence of reactions.

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When the presence of the label is confirmed at the end of the test, it can be concluded that an antigen–antibody reaction took place.

Techniques using a label as an indicator include the following:

- Enzyme linked immunosorbent assay (ELISA) – the antibody is labelled with an enzyme that brings about a colour change when an enzymatic substrate is added.
- Radioimmunoassay (RIA) – before the development of ELISA tests, the antibody was labelled with a radioisotope that produced a signal that could be measured.
- Immunofluorescence – the antibody is labelled with a fluorescent dye that is visible by its ability to reflect light.

The commonly used ELISA methods are described in *Section 4: Principles of laboratory techniques.*

Role of complement

The role of complement was introduced in *Section 2: Immunology*. Complement may participate in antigen–antibody reactions *in vitro* in two ways; either by red cell adherence, or by causing haemolysis. The nature of the antigen–antibody reaction determines its involvement.

Complement (C′) is a large group of nine proteins present in abundance in the body, and in freshly drawn serum samples. Complement from one species is effective in antigen–antibody reactions in many other species. When activated, a cascade starts, and amplifies. One molecule of C1 attached to the cell wall, results in hundreds of molecules of C3 being activated.

Summary of classical pathway of complement cascade

When complement-binding antibodies react with their corresponding antigens, the fragment crystallizable (Fc) portions undergo a change that attracts the C1 component of complement. C1 is a large molecule consisting of C1q, C1r and C1s that when combined with Fc, inter-react and cause further changes and cleavage of C1. The complex C1 component is stabilized in the presence of calcium ions (Ca^{++}), without which C1 dissociates and becomes inactive. (This explains why anticoagulants that chelate calcium are anticomplementary.) Activated C1s cleaves C4 into C4a and C4b. Some C4b also becomes cell bound, and forms the binding site for C2. C1s also cleaves C2 into C2a and C2b. Whereas C2a remains cell bound, C2b dissociates from the cell wall. This happens in the presence of magnesium ions (Mg^{++}). This part of the cascade results in an activated complex of C4b2a, also called C3 convertase. (Some authorities now believe that C2a dissociates, and C2b remains cell bound, forming C4b2b, which is the reverse of the above.) C3 convertase cleaves hundreds of molecules of C3 into C3a (which dissociates from the cell) and C3b, which becomes cell bound. C3 convertase (C4b2a3b) cleaves C5 into C5a and C5b. C5b then binds C6, C7, C8 and C9, which results in the cell membrane being pierced. When C8 is cell bound, lysis is slight; when C9 is cell bound, lysis is pronounced. C3 convertase can progress to the membrane attack stage, or inhibitors can stop the process, which then ends with C3b coating the cell wall. Cells coated with complement *in vivo* are removed from the circulation for destruction in the liver and/or spleen. Figure 3·7 shows the chain reaction of the complement cascade in detail.

In the laboratory, complement plays several roles:

- Haemolysin test – used to demonstrate the presence of immune ABO antibodies that utilize complement when they react with red cells expressing the corresponding ABO antigens by causing activation all the way to membrane attack and subsequent haemolysis.
- Direct or indirect antiglobulin test: used to demonstrate the presence of complement-binding antibodies attached to red cells with the corresponding antigens, causing the activation of complement, but not all the way to its membrane attack stage. Instead, the complement cascade stops when it becomes cell bound. This is referred to as complement fixation, which is detectable using an antiglobulin reagent that contains anti-complement antibodies and will therefore agglutinate cells with complement coating their surfaces.

Factors that influence the participation of complement in laboratory tests

Chemicals

- Clotted samples are ideal for tests involving complement. However, most blood samples today are taken into anticoagulant, and anticoagulants such as citrate, EDTA and oxalate are anticomplementary, as they chelate or bind calcium. This means that they block complement participation in antigen–antibody reactions. The complement cascade needs calcium to proceed, and if calcium is chelated or blocked, it cannot be used. Therefore, anticoagulated plasma samples should not be used for haemolysin tests or to screen for complement-binding antibodies in antiglobulin tests or to carry out complement utilization studies.
- Heparin is an example of an anticoagulant that does not chelate calcium, so heparinized samples may be used for specific tests.
- Reagent red cells are suspended in preservation fluid rather than normal saline, so that they may be used over a period of time (i.e. 6 weeks) in the laboratory. However, this preserving fluid may also be anticomplementary, so if the reagent cells are required for haemolysin tests, they should be washed and then resuspended in saline.

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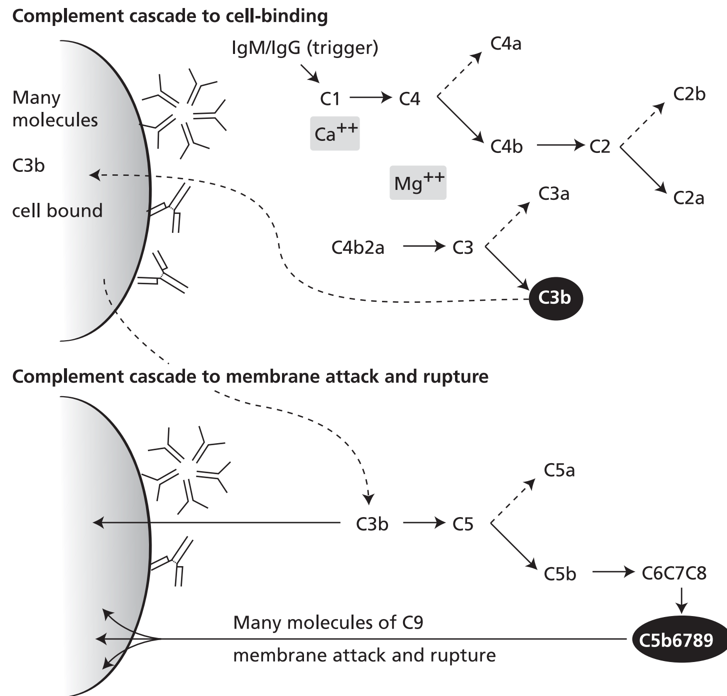

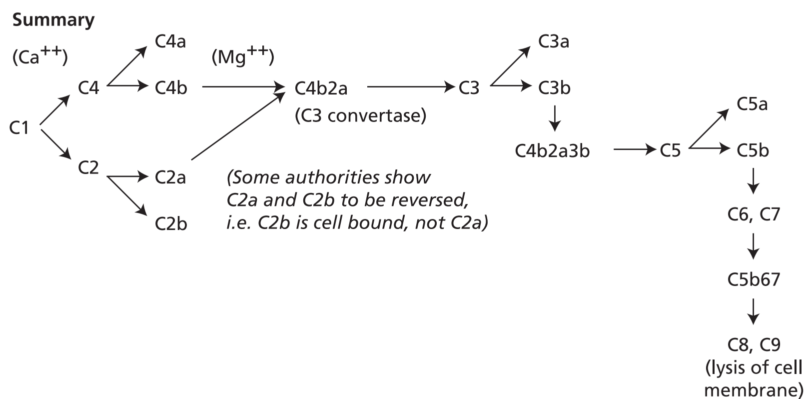

Fig. 3·7 Detail of complement cascade.

Time and temperature

- When a clotted blood sample is taken, it contains complement but this deteriorates steadily. Its properties are also affected by the surrounding temperature; the higher the temperature, the quicker the deterioration. Complement is said to be labile because it easily loses its active characteristics.
- Serum samples stored in the refrigerator at +2°C to +10°C lose their complement activity after 24 hours. Serum samples stored frozen at –18°C or colder will not lose their complement activity for several weeks, provided that the sample is frozen while fresh (within a few hours of being taken). Complement activity may be deliberately removed from serum by placing it at a temperature of +56°C for 30 minutes.
- If the test to be performed requires complement, either to demonstrate haemolysis or complement-binding, and the serum to be tested is aged, then complement from an external source may be added.

Role of antihuman globulin

In 1945 Coombs and workers introduced a test that had been developed many years earlier, for detecting weak, incomplete (sensitizing, IgG) antibodies. Previously known as the Coombs test, it is now referred to as the antiglobulin test.

Historically, AHG was prepared by injecting animals of another species, like rabbits or goats, with human proteins. The two types of human proteins that were introduced as antigens into the animals were:

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- Immunoglobulin G
- Complement.

Several animals were used; the one batch injected with IgG and the other with the C3 component of complement. Because these human globulins/proteins are foreign to the animals, their immune system was stimulated to respond, not only to human species proteins, but specifically to IgG and complement proteins, producing antihuman species (heterophile) antibodies but also antibodies specific to IgG and C3.

After an appropriate response time, samples of blood were drawn from the animals, and after adsorption of the anti-species antibodies, their serum/plasma examined for the presence of anti-IgG and/or anti-C3 component of complement. To prepare a broad spectrum reagent, a blend or pool of the best anti-IgG and anti-C3 was processed and standardized for routine use in the laboratory.

The purpose of a broad spectrum or polyspecific AHG reagent is to detect and agglutinate cells, which have been sensitized by human IgG or C3 or both. (In order to simplify the anticomplement activity, in this publication the term anti-C3 is used to signify predominantly anti-C3d, with or without the presence of anti-C3b) By introducing AHG into the test, an antigen–antibody reaction will occur between the cell bound globulin/protein and the antiglobulin reagent. The AHG is able to bridge the gap between adjacent sensitized cells, agglutinating them and making the original reaction (between red cell antigens and specific antibodies, with or without the involvement of complement) visible.

Most AHG used in laboratories today is commercially manufactured monoclonal AHG and is readily available as blends of anti-IgG and anticomplement or may be monospecific for either IgG or complement.

Washing the cells after their incubation with serum/plasma, and prior to the addition of the antiglobulin reagent, is crucial to the success of any AHG test. It is important to remove all unbound globulin from the cells to be tested prior to the addition of AHG. If the cells are not adequately washed, unbound globulins or complement components are able to react with AHG and in so doing neutralize it, rendering it inactive and unable to combine with cell bound material. This leads to false negative results and demonstrates the importance of controlling AHG tests that are found to be negative. For additional information, refer to *Section 4: Principles of laboratory techniques.*

The reaction between globulin and antiglobulin is specific in that the AHG recognizes human globulin, or complement, or both. It does not recognize the actual specificity of the antibodies, which originally reacted with the antigens on the red blood cells.

Unlike proteolytic enzymes and high protein media, AHG does not reduce zeta potential, but bridges the gap between sensitized cells.

Figure 3·8 describes and depicts the action of AHG on IgG and complement sensitized red cells. The last picture in the figure shows the involvement of both components of broad spectrum AHG.

Application of antiglobulin tests in the laboratory

Direct antiglobulin test

The direct antiglobulin test (DAT) is performed on red cells to determine whether they were sensitized *in vivo*. The serum/plasma in the sample does not play a role in this test, and there is no incubation stage. The test involves immediate washing of patient's red cells, followed by the addition of AHG. A positive result indicates that the patient's cells are sensitized in the body and this has the potential to cause *in vivo* haemolysis. A positive DAT can be the result of a number of causes, including haemolytic disease of the fetus and newborn, haemolytic anaemia caused by autoantibodies or induced by drugs, the sensitization of transfused red cells, as the result of an incompatible blood transfusion or various diseases.

There are numerous other *in vitro* uses for the DAT, such as to confirm that laboratory prepared red cells for use in tests to detect IgG antibodies are unsensitized prior to use.

A DAT performed on a refrigerated clotted blood sample may give false positive results should broad spectrum antiglobulin reagent be employed. This is because it is quite likely for complement to have become red cell bound in the refrigerator, as a result of the presence of cold antibodies, not necessarily of clinical significance. For this reason, many laboratories prefer to use an anticoagulated sample (i.e. a sample in which the complement was inactivated at the time of collection) for the DAT.

Indirect antiglobulin test

In the case of the indirect antiglobulin test (IAT), serum/plasma and cells are incubated in the laboratory to allow sensitization to take place. Only then are the cells washed to remove unbound globulins, before the addition of AHG. Agglutination indicates that an antigen–antibody reaction took place during the incubation phase.

When the IAT is carried out, usually the cells or the serum/plasma is of known characteristics. For example, one may use an IgG typing reagent in order to establish whether unknown cells have the corresponding antigen or not. This is known as red cell typing. On the other hand, one may use reagent red cells of known antigen type to detect unexpected antibodies in serum/plasma with unknown properties. This is known as antibody screening or antibody identification.

It is preferable to use fresh serum for the detection of complement-binding antibodies in the IAT. If an antibody of this nature is very weak (i.e. if it has low affinity), then it may be dislodged from the cells after incubation during the

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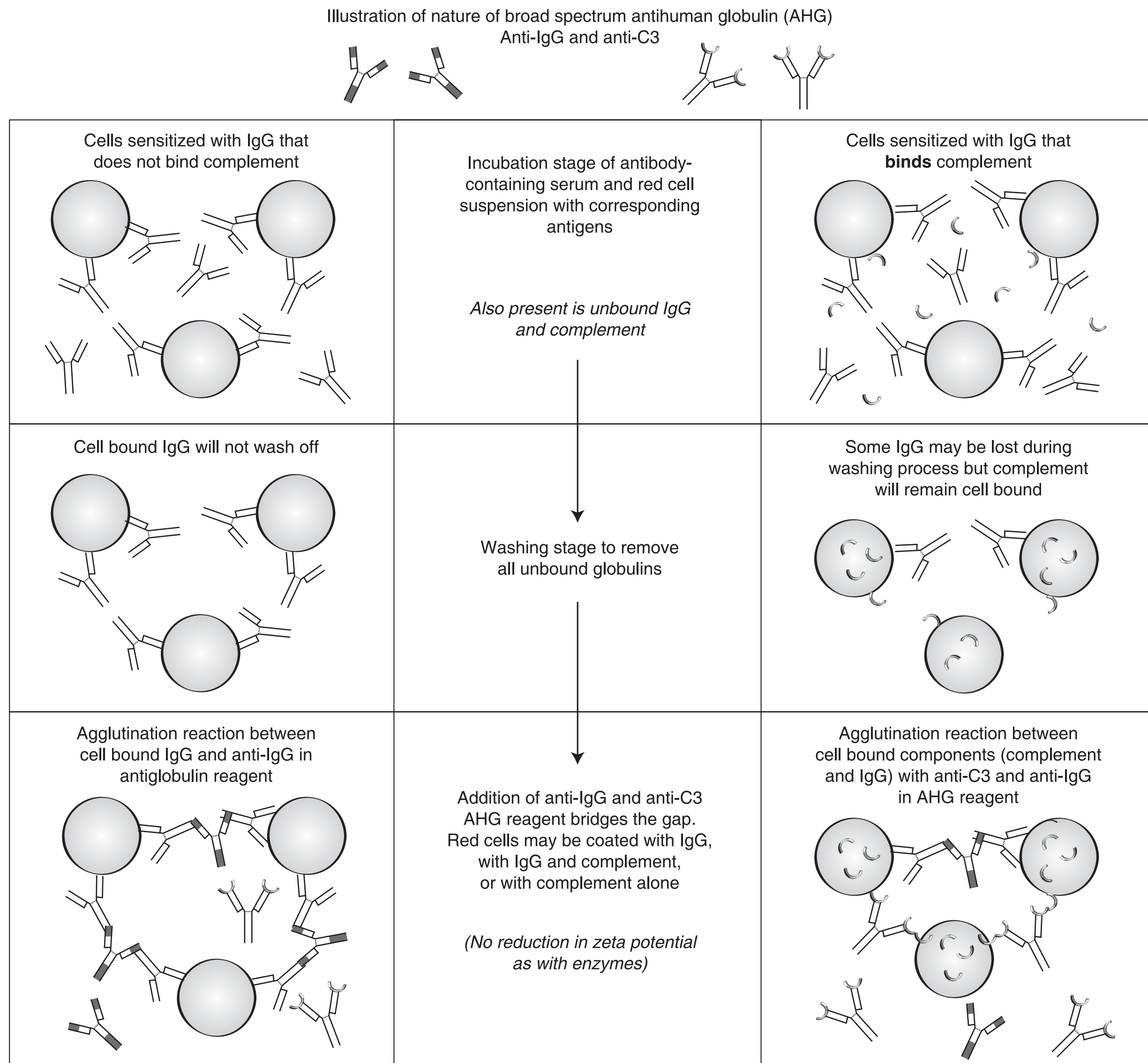

Fig. 3·8 Action of broad spectrum AHG on IgG and complement coated red cells.

washing process. However, if complement became cell bound, it will not wash off the cells. Use of AHG reagent containing anti-C3 will cause these complement bound cells to agglutinate, indirectly indicating that an antibody was present.

Polyclonal and monoclonal antibodies

Polyclonal antibodies

Polyclonal antibodies are usually of human origin. Polyclonal is a term used to describe antibodies that have the same specificity, but are produced by different clones of antibody producing cells, and each antibody molecule is therefore not exactly the same, although they react with the same epitope. One may liken polyclonal antibodies to several keys that have very similar structure and are therefore all able to fit the same lock.

For example, anti-A is present in all group B individuals who have a normal immune system. Yet the anti-A produced in one individual is not *exactly* the same as the anti-A produced in another. This applies equally to other antibodies produced by humans. Because of this individual variation, batches of anti-A (or anti-B, etc.) prepared from humans, had to be standardized every time that a new batch was produced, to ensure continuity of reaction behaviour and strength from batch to batch and to identify any possible contaminating

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antibodies of other specificities. Because of the use of monoclonal antibodies as grouping reagents in the laboratory today, variation of this nature is no longer a challenge.

Monoclonal antibodies

Monoclonal antibodies are produced *in vitro*, using hybridoma technology. They are produced from a single immune cell line, therefore all of the antibody molecules produced are exactly the same. There is no variation from batch to batch prepared under identical conditions. The starting cell line may be of murine (mouse) or human origin.

Outline of process to produce murine monoclonal antibody

1. Mice are immunized with human red cells containing the antigen corresponding to the specificity of antibodies to be produced (e.g. A cells to produce anti-A).
2. After immunization, mouse spleen cells are removed and fused with human myeloma cells (plasma cells that have become malignant and can be continuously cultured *in vitro*).
3. After fusion, some of the cells produced are a mixture of mouse and myeloma cells – these are called hybridomas.
4. Hybridoma cells are allowed to grow for some time and then tested for antibodies.
5. Those hybridoma cells that are good antibody producers are selected and cloned (clones are reproduced from a single cell and are identical in their inherited characteristics).
6. In time, clones grow into colonies of monoclonal cells (all the same).
7. Antibody content is measured again, and colonies producing good antibodies are selected and stored frozen.
8. These colonies can later be retrieved to produce large-scale tissue cultures, secreting monoclonal antibodies of exactly the same specificity.
9. This allows for the production of vast quantities of high titre monospecific antibody for ongoing laboratory use.

Advantages

- Availability of large quantities of monospecific reagent antibody that reacts in a reproducible way
- No variation from batch to batch. Antibodies are produced in pure form, whereas antibodies of human origin are polyclonal.

Disadvantages

- The generation and successful recovery of stable, cloned hybridomas is time-consuming
- The process requires special laboratory equipment and storage facilities.

Figure 3·9 is a diagram that shows the production of mouse monoclonal antibodies by hybridoma.

Laboratory reagents such as anti-A and anti-B were originally of human origin. Antiglobulin was originally produced in animals. These reagents, being polyclonal and subject to batch variation, had to be carefully standardized every time a batch was produced. With the development of monoclonal antibodies that lack variation, it is not surprising that they are now the reagents of choice. Unlike human polyclonal antibodies, monoclonal antibodies are highly specific and of high titre. They are not subject to interference with non-specific cross-reactions like their polyspecific counterparts. Because of their high affinity for antigen, they are able to react strongly even with weakly expressed antigens. Commercially prepared type IgM, IgG and various blends of monoclonal antibodies are now commonly used in blood grouping and antiglobulin tests.

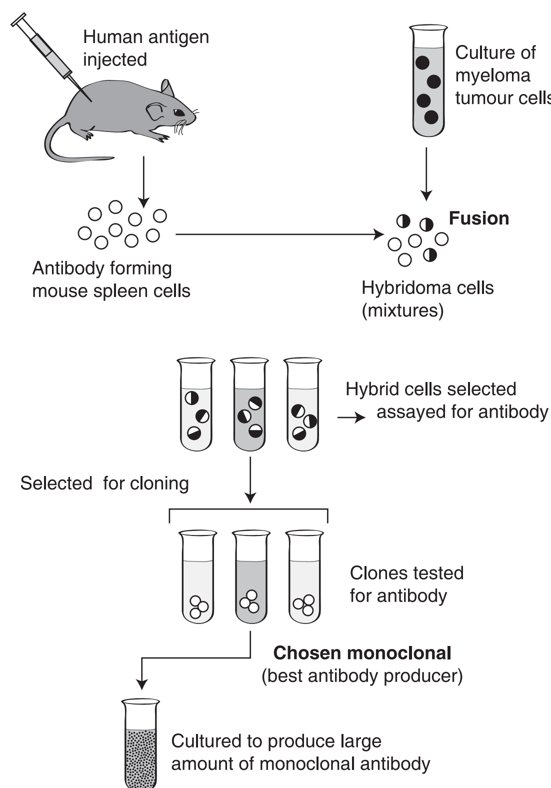

Fig. 3·9 Production of mouse monoclonal antibodies by hybridoma.

Summary of section: Antigen–antibody reactions

- Antigen–antibody reactions occur in two stages; the first is rapid and the second takes time for the reaction to become demonstrable.
- Centrifugation is the most widely used way to enhance antigen–antibody reactions.
- Haemagglutination occurs when IgM antibodies react with their corresponding red cell antigens.
- Sensitization occurs when IgG antibodies react with their corresponding red cell antigens.

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- Sensitization is not an observable reaction, and potentiators may be employed to allow sensitized cells to agglutinate.
- Haemolysis is the result of antigen–antibody reactions utilizing the complement cascade all the way to cell membrane attack and rupture.
- Neutralization of antibody occurs in the presence of the corresponding antigen in soluble form. An antibody that has been neutralized cannot thereafter react with red cells containing the corresponding antigen.
- Precipitation of soluble antigen and antibody is able to take place when the two reactants are present in the correct proportions. Alternatively, immunodiffusion allows for the development of a precipitin line between antigen and antibody, in an appropriate gel medium.
- Many factors influence antigen–antibody reactions; these include the number and site of antigenic determinants on cells, the distance between epitopes, the electric repulsion between red cells, the goodness of fit between antibody and antigen, the immunoglobulin class, the concentration of antibody, as well as the effects of time, temperature, pH and ionic strength of the surrounding test environment.
- Proteolytic enzymes are able to reduce zeta potential, causing sensitized cells to agglutinate.
- Enzymes may be used in one- or two-stage techniques; it is important to note, however, that some antigens are modified by enzymes and that their corresponding antibodies will therefore not be detectable in an enzyme medium.
- The enzymes in laboratory use include bromelin, ficin, papain and trypsin.
- High molecular mass substances such as albumin, polyethylene glycol, and polybrene, are also able to affect zeta potential and cause the agglutination of sensitized cells.
- Instead of normal ionic strength saline (NISS), low ionic strength saline (LISS) is commonly used in antibody detection tests because incubation time is reduced and antibody uptake is increased. Therefore, the sensitivity of antigen–antibody reactions is enhanced.
- Complement is a serum/plasma protein complex that amplifies antigen–antibody reactions. When involved in antigen–antibody reactions, it either leads to lysis, or complement fixation (binding to cell walls).
- *In vitro*, complement is labile and adversely affected by time and temperature. Its action is also prevented by anticoagulants, which block calcium, an ingredient required in the complement cascade.
- Antihuman globulin (AHG) causes the agglutination of sensitized cells by bridging the gap between them. AHG may be monospecific, or broad spectrum, containing antibodies to both IgG and complement.
- The direct antiglobulin test will detect red cell sensitization *in vivo*.
- The indirect antiglobulin test is used to determine whether an unknown serum/plasma sample contains IgG antibodies or to determine, when using an IgG grouping reagent, whether a red cell sample contains a particular antigen.
- Monoclonal antibodies are produced using hybridoma technology. Each monoclonal antibody is produced from an 'immortal' single cell line and produces antibody of exactly the same specificity every time. There is therefore no need for comprehensive standardization from batch to batch in the blood bank, as for human polyclonal antibodies.

Additional learning activities

(1) It is suggested that students use a medical dictionary and/or the Internet to clarify the meaning of words and phrases and to add to the information provided in this section. A list of key words that may be useful in this regard is provided below.

- Agglutination
- Sensitization
- Haemolysis
- Immune precipitation
- pH
- Proteolytic enzymes
- Albumin
- Complement system
- Coombs test
- Monoclonal antibodies

(2) Communicating via the person in charge of the laboratory, share information with other students/readers with regard to the use of potentiators in laboratory tests. What are the advantages and/or disadvantages of the different potentiators? Are monoclonal antibodies used for determining blood groups? Is there a role for polyclonal human reagents for any laboratory tests?

Conflicts of interest

The contributing author of this section on Antigen–antibody reactions (BA) has not received grants, speakers fees etc., from any commercial body within the past 2 years. The author has no potential conflicts.

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