Investigation of patients with autoimmune haemolytic anaemia and provision of blood for transfusion

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Introduction

Autoimmune haemolysis may be defined as a reduced red cell lifespan due to the production of antibodies which react with antigens carried on the individual's own red cells. When the rate of red cell destruction exceeds the regenerative capacity of the bone marrow, anaemia results. Autoimmune haemolytic disease may be conveniently classified into warm, cold and mixed types reflecting the thermal optima of the autoantibodies responsible.¹ It may occur as a primary condition or be associated aetiologically with other diseases, notably lymphoproliferative disorders, other autoimmune conditions (for example, systemic lupus erythematosus, ulcerative colitis), myelodysplastic syndromes or certain drugs—methyl-dopa being the classic example.¹ A rare type of autoimmune haemolysis can occur if the red cell membrane is altered to expose certain cryptantigens (for example, T and Tk) which then react with specific antibodies present in the serum of most individuals.²

Red cell autoantibodies are of IgG, IgM and IgA classes. Warm autoantibodies are mainly IgG, although recent studies using sensitive techniques have shown that some 37% of cases also have increased amounts of IgM or IgA, or both, bound to the red cells.³ Cold autoantibodies are usually IgM, the notable exception being the IgG class Donath-Landsteiner antibody; however, examples of cold reacting IgA autoantibodies (for example, anti-Pr) have also been reported.⁴

In patients with autoimmune haemolysis, the coating of autoantibodies per se does not damage the red cells but causes haemolysis via complement activation and/or by inducing interactions with effector cells, mainly in the mononuclear phagocyte system.¹⁻⁵ Haemolysis may be extravascular (more common) or intravascular (more spectacular).

Extravascular haemolysis occurs when autoantibodies or C3 components bind to red cells and react with specific receptors on mononuclear phagocytes (or other white cells) resulting in red cell destruction through phagocytosis, spherocyte formation or antibody

dependent cellular cytotoxicity. The different immunoglobulin classes and complement components may act synergistically in bringing about red cell destruction.³

Intravascular haemolysis occurs when complement is fully activated. It is rare (being seen in less than a fifth of patients with autoimmune haemolytic anaemia), though it is more common in those cases occurring in childhood.¹ Autoantibodies which trigger intravascular haemolysis are mostly of IgM class, but it can also be caused by some warm reacting antibodies of IgG1 and IgG3 subclass; the most important IgG example, however, is the cold reacting Donath-Landsteiner antibody.¹ Traditionally, IgA class red cell autoantibodies are thought not to activate complement, but this may not always be the case as there are scattered reports of patients with autoimmune haemolytic anaemia and intravascular haemolysis where only IgA class antibodies were detected⁶; a suggestion has been made that IgA antibodies can activate complement when they are in an aggregated form.^{6,7} Complement activation is often triggered, but rarely proceeds beyond the C3 stage because of the presence of regulatory inhibitors. Red cells circulating with C3b bound to their surface are removed by phagocytosis, mainly by the liver macrophages. The naturally occurring regulatory factors act on any red cells not engulfed, cleaving the C3b to C3d,g; these cells survive normally and are identified in vitro with anti-C3d reagents.¹

Diagnosis of autoimmune haemolysis is based on demonstrating that autoantibodies or complement components, or both, are bound to the red cells and are associated with a shortened red cell lifespan. Diagnosis may be easy or, on occasions, be extremely difficult—for example, when it is a minor part of a chronic disorder or serious condition where the effects of haemorrhage, treatment, blood transfusions, and other causes of anaemia, have also to be taken into account.¹

This broadsheet is based on the standard operating procedures used at the Trent Blood Transfusion Centre (Sheffield) for investigating suspected cases of autoimmune haemolytic

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Investigation of patients with autoimmune haemolytic anaemia. * Tests considered to be more appropriate for specialist laboratories.

anaemia. It aims to provide guidance to immunohaematological tests which can be carried out in a hospital blood bank, as well as giving an overview of some of the techniques which can be performed in a specialist laboratory. Using these guidelines, summarised in the figure, even serologically complex cases can be unravelled and the patients safely transfused.

Samples required

The investigation of autoimmune haemolytic anaemia can be complicated and therefore it is important that adequate blood samples are obtained from the patient. Full testing can then be performed without delay, thus allowing any blood cross-matched to be transfused with confidence that it will not harm the recipient.

Our ideal samples are, firstly, serum from 30 ml clotted blood; this should be separated at 37°C to permit accurate assessment of the thermal amplitude and titration score of any cold autoantibodies present. Both amplitude and score will be artefactually reduced if the samples are allowed to clot at room temperature or placed in a refrigerator at 4°C as the cold autoantibodies will be adsorbed on to the red cells.

Secondly, we like 20 ml blood collected with EDTA as anticoagulant—these samples are required for grouping, direct antiglobulin tests, the production of eluates, and for auto-absorption studies. Their use for direct antiglobulin testing is strongly recommended as

EDTA inhibits in vitro complement activation and therefore any complement components detected on the red cells will be the result of activation in vivo.⁸

Finally, an "Investigation Request Form" is required; this should give full personal and clinical details, including drug therapy, previous pregnancies and transfusions and the results of any relevant laboratory tests.

Tests carried out on patients' red cells

ABO GROUPING AND Rh GENOTYPING

ABO grouping is part of any standard serological investigation of autoimmune haemolysis. It becomes particularly important if transfusions are required, or if rare A or B specific autoantibodies are suspected. Although standard saline agglutination procedures are used, ABO grouping may be difficult in the presence of large amounts of autoantibodies and control tests using inert AB serum and monoclonal control media must be included. If group A subtyping is required, it is best performed using the lectin *Dolichos biflorus* (which shows anti-A₁ specificity). Cold autoagglutinins are a particular problem and the patient's red cells may have to be washed several times in phosphate buffered saline (PBS) (pH 7.0) prewarmed to 37°C before they are suitable for grouping; it may even be necessary to ABO group at 37°C as well as at 18°C. Reverse or serum grouping with group A₁, A₂, B, and O cells as well as with the patient's own red

cells is an important part of the procedure, and may also have to be performed at 37°C.

Full Rh genotyping is carried out so that suitable blood can be selected for transfusion and also to permit any red cell antibodies showing Rh specificity to be identified as either auto- or alloantibodies. It is important that "saline agglutinating" reagents are used as patients' red cells which are heavily coated with immunoglobulins may spontaneously agglutinate if suspended in solutions of bovine albumin or proteolytic enzymes. The red cells should be washed four times in PBS (pH 7.0) (prewarmed to 37°C if cold autoagglutinins are suspected) before Rh typing. A 3–5% suspension of red cells is tested for the C, D, E, c, e, and C^w antigens. Suitable control cells must be used for each reagent, and inert AB serum or monoclonal control media, or both, must be included to identify any autoagglutination. Commercially available IgM monoclonal Rh antisera give fast and dependable results; they usually require a short incubation at room temperature followed by gentle centrifugation. The results are read microscopically.

In some patients with autoimmune haemolytic anaemia the cells are so heavily coated with immunoglobulin that spontaneous agglutination makes it impossible to type the cells using the above methods. In these cases chloroquine can be used to remove red cell bound immunoglobulin.⁹ Suitable control cells must also be treated as prolonged exposure to chloroquine may damage the red cell antigens. ZZAP, a mixture of dithiothreitol and cysteine activated papain,¹⁰ has been recommended as an alternative to chloroquine, but has the disadvantage of denaturing Kell, Duffy and MNS antigens.

Method using chloroquine—The patient's and control cells are washed three times in PBS (pH 7.0); 5% suspensions in chloroquine diphosphate solution (200 mg/ml in PBS (pH 5.0)) are incubated either at 30°C for 90 minutes or at 37°C for a maximum of 30 minutes, washed twice and typed.⁹

DIRECT ANTIGLOBULIN TESTS

Direct antiglobulin tests are used to detect increased amounts of red cell bound immunoproteins. A positive test result may be due to (i) autoantibodies and complement components, (ii) non-specific adsorption of protein—for example, in patients with myeloma or in those with a general increase in γ -globulin concentrations, (iii) immune complexes, (iv) drug induced antibodies, and (v) alloantibodies—for example, in haemolytic disease of the newborn or when the transfusion of incompatible blood has resulted in a delayed transfusion reaction.

In the standard agglutination direct antiglobulin test a range of antisera is employed. Broad spectrum reagents are valuable for initial screening and must contain at least anti-IgG, -IgA and -C3d. Monospecific reagents are used to identify which immunoproteins are coating

the red cells: anti-IgG, -IgA, -IgM, -C3d, -C4c, and -C3c, as well as IgG subclass antisera, are now commonly used. The anti-immunoglobulin reagents must be heavy chain specific. In our experience a good anti-IgG will start to give positive results at about 150 molecules per red cell.

Method—Red cells are washed at least four times in PBS (pH 7.0) and a 2% suspension is tested using a spin tube technique (one drop of red cell suspension to two drops of antiglobulin reagent). The results are read microscopically. Where low affinity antibodies are suspected, ice-cold PBS (pH 7.0) is used to wash the red cells before testing.

Direct antiglobulin tests utilising enzyme linked reagents may also be useful in the investigation of patients with autoimmune haemolytic anaemia.^{11–13} These methods are extremely sensitive, being able to detect the small quantities of immunoglobulins found on normal red cells (about 33 molecules per cell in the case of IgG)¹³; they can thus recognise slightly increased amounts of cell bound autoantibodies (particularly of IgM and IgA class) which, although undetectable by agglutination techniques, may have a significant clinical effect.^{3,12,13}

Method—Alkaline phosphatase linked anti-IgG, -IgA and -IgM (50 μ l) (Sigma, Poole, Dorset, UK), appropriately diluted in PBS (pH 7.0), are mixed with 25 μ l of a 25% suspension of washed test and control red cells in a U-well microtitre plate. After incubation at 37°C, the cells are washed six times in PBS. Substrate (200 μ l) (P-nitrophenyl phosphate 1 mg/ml in carbonate buffer (pH 9.8)) is added to each well and the plate incubated at 25°C for a further 25 minutes. The plate is centrifuged and 100 μ l of supernatant from each well is transferred to a flat-well microtitre plate, the reactions are stopped using 50 μ l 3 M NaOH and the optical densities read at 405 nm. Red cell counts on the buttons of cells left in the U-well plate allow the optical density readings to be adjusted to a standard cell count. A result is considered to be positive when the optical density value is more than 3 SDs above the mean value obtained for a series of healthy subjects.¹³

A number of promising new methods which utilise column technology are coming on to the market. In the best known example (DiaMed AG, Cressier sur Morat, Switzerland) cards are manufactured containing a set of monospecific antiglobulin reagents in gel. The red cells (which require no washing as serum or plasma cannot enter the gel and neutralise the reagents) are diluted in low ionic strength saline (LISS), added to wells at the top of each column and centrifuged. Agglutinated red cells are held in the gel matrix whilst non-agglutinated cells form a button at the bottom of the column. The results are read visually and can be photocopied as a record. The lack of a washing

step improves the sensitivity of the direct antiglobulin test and also permits detection of low affinity autoantibodies. At the time of writing, our experience with the DiaMed system is that the anti-IgG is excellent (with a sensitivity between the agglutination and enzyme linked methods), but the anti-C3d and -IgM are less satisfactory. In fact, the latter is now being replaced by an anti-IgA reagent.

TESTS ON RED CELL ELUATES

These are carried out to elucidate the cause of a positive direct antiglobulin test. Autoantibodies, for example, rebind to normal red cells and the immunoglobulin concentration which occurs during preparation makes eluates especially suitable for determining the immunoglobulin class, subclass and any blood group specificity. Direct antiglobulin tests which show large amounts of cell bound IgG, but where no antibody is found in the eluate, may be due to drugs such as penicillin. Other examples of a positive direct antiglobulin test and no elutable antibody are seen in patients with increased amounts of immune complexes or those with paraprotein or γ -globulins non-specifically coating the red cells. If a specific antibody is eluted, the patient's red cells should be checked for the blood group concerned. The presence of alloantibodies in an eluate suggests a delayed transfusion reaction (or haemolytic disease of the newborn), though "mimicking" autoantibodies^{14,15} and the Matuhasi-Ogata phenomenon¹⁶ where alloantibodies can be found non-specifically associated with autoantibodies in red cell eluates should not be forgotten when interpreting the results.

There are many different techniques for producing eluates; two which we have found particularly useful will be described. The chloroform/trichloroethylene method¹⁷ is suitable for red cells which are strongly IgG sensitised (that is, those giving a visible reaction in the standard direct antiglobulin test).

Method—Packed red cells (1 ml) are washed four times and resuspended to 50% in PBS (pH 7.0). Two volumes of chloroform/trichloroethylene mixture (1:1) are added, the tube is sealed and the contents mixed vigorously. The tube is then incubated at 37°C for 10 minutes with occasional mixing and then centrifuged at high speed for five minutes. The eluate is the haemoglobin stained supernatant.

We recommend using acid elution methods if the direct antiglobulin test is only weakly positive (suggesting a small increase in cell bound immunoglobulins). These techniques have the advantage of causing minimal damage to the red cell membrane. They are based on the principle that reducing the pH breaks down the bonds between antibody and red cell. Being free from haemoglobin, acid eluates can be concentrated (for example, with a Minicon-CS15 spinal fluid concentrator) to permit detection of weaker antibodies. The ELU-PLUS (Dominion Biologicals, Dartmouth, Nova

Scotia, Canada) is an excellent commercial red cell acid elution kit.

Method—Packed cells (1 ml) are washed four times in the "wash solution" provided and 1 ml glycine HCl buffer solution (pH 3.0) incorporating a pH indicator is added. The tube is sealed, mixed and centrifuged immediately for one minute, the supernatant eluate being taken off without delay. The pH is adjusted to 6.5–7.5 (shown by blue colour development) by dropwise addition of the "base solution"; any precipitate which forms is removed by centrifugation.

Eluates prepared using either of the methods described above are tested by an indirect antiglobulin technique for antibody specificity against a short panel of group O red cells fully typed for the most common blood group antigens. The immunoglobulin class and IgG subclass of the autoantibody are determined by incubating the eluate with red cells for one hour at 37°C, washing and then testing with monospecific anti-IgG, -IgM, and -IgA as well as with anti-IgG subclass reagents.

TESTS FOR POLYAGGLUTINABLE RED CELLS

Polyagglutination² has on rare occasions been associated with autoimmune haemolytic anaemia. Many of the acquired forms, such as T, Tk, acquired B, Th, and VA, are produced by the action of microbial enzymes on the red cell membrane, altering its structure and exposing cryptantigens. Naturally occurring IgM antibodies in normal adult (and in certain animal) serum may react with these cryptantigens causing red cell destruction,^{2,18} however, some authorities believe that in most, if not all cases of polyagglutination, the haemolysis is caused by the action of the microbial enzymes rather than the antibodies.¹⁹

Polyagglutinable cells are agglutinated by a large proportion of ABO compatible adult human serum samples, but not by cord or (usually) by autologous serum, and sometimes give variable non-specific reactions with anti-human globulin reagents. Suspected polyagglutinable cells should therefore be mixed (as a 3–5% suspension in saline) with several group AB adult and cord serum samples. If agglutination is observed with the majority of the adult samples, but not with the cord samples, polyagglutination is likely. The various types of polyagglutination can be easily differentiated by use of a small panel of lectins (Gamma Biologicals, Houston, Texas, USA) which includes extracts of *Arachis hypogaea*, *Salvia sclarea*, *S. horminum*, and *Glycine Soja*.

FUNCTIONAL CELLULAR ASSAYS

These tests can be useful in evaluating the clinical significance of autoantibodies although they are rarely carried out routinely.

The monocyte monolayer assay has been used most often.²⁰ It involves producing a layer of monocytes by adherence, incubating with a suspension of patient's red cells and, after fixing

and staining, counting the percentage of monocytes with phagocytosed or adherent red cells, or both. The test is simple to perform; either donor or autologous monocytes can be used, with perhaps the patient's own monocytes being more representative of the *in vivo* situation.

Antibody dependent cellular cytotoxicity assays have rarely been used in the investigation of autoimmune haemolysis.²¹ They assess the amount of cytotoxic damage to ⁵¹Cr labelled red cells by measuring the amount of isotope released by effector cells, usually monocytes (or lymphocytes). The test is quantitative, though has the disadvantages of being difficult and expensive to perform because of the use of radioisotopes. Introduction of a method using enzyme linked labels would be an interesting future development.

The chemiluminescence test has not been evaluated in patients with autoimmune haemolytic anaemia as yet.²² It assesses the monocyte response of immunoglobulin coated red cells during phagocytosis: the monocytes produce oxygen radicals which react with luminol generating light emission which is measured in a luminometer. The test is simple, rapid and objective, and would seem to have potential value in assessing the clinical significance of red cell autoantibodies.

Tests carried out on patients' serum

ANTIBODY INVESTIGATIONS

The purpose of these tests is to detect auto- and alloantibodies in the patients' serum, to determine whether the autoantibodies are of warm or cold type and to identify blood group specificity. Each patient's serum is tested against their own red cells (auto-control) and a panel of group O cells which have been fully typed for the common blood group systems (Rh, Kell, Duffy, Kidd, Lewis, MNS, and P) using saline agglutination at 18°C and 37°C and albumin, papain and indirect antiglobulin techniques at 37°C. Although these are standard serological methods, it is most important to pay meticulous attention to detail. Serum and red cell suspensions should be warmed before mixing. The results should be read microscopically, making careful note of reaction strengths as variations may indicate a mixture of auto- and alloantibodies. If cold autoagglutinins with a wide thermal range are present, the results of tests performed at 37°C should be read on slides prewarmed to that temperature, although the cold antibodies may not be active at 37°C. If the tests are allowed to cool before being read, then agglutinates will form which make the interpretation of the 37°C results inaccurate. Because of the problems with cooling, indirect antiglobulin tests may benefit from suspension in PBS, prewarmed to 37°C, before the normal wash cycle.

The albumin displacement technique using 20% bovine albumin, although outmoded by more sensitive methods, is included both for the detection of alloantibodies when the autoantibody reactions with enzyme and indirect antiglobulin techniques are very strong and also for identifying gross rouleaux formation which can be mistaken for autoantibody.

Indirect antiglobulin tests using LISS are utilised as they are sensitive and give rapid results. They are based on the principle that decreasing the ionic strength of a reaction mixture increases the rate at which antigen-antibody complexes are formed. Some patients, however, have non-clinically significant autoantibodies which react only when using the LISS method, giving negative reactions with indirect antiglobulin techniques utilising isotonic saline. In our experience these cases, which usually have a negative or weakly positive direct antiglobulin test, often have increased γ -globulin concentrations, and show weak rouleaux formation in the saline and papain tests.

EXAMINATION OF THE SPECIFICITY AND THERMAL RANGE OF COLD AUTOAGGLUTININS

In cases where there is a cold autoagglutinin of possible clinical significance the serum is titrated in saline (doubling dilutions from neat to 1 in 512 are usually sufficient) and tested with pooled OI, Oi, papainised OI, and the patient's own red cells at 18°C and 30°C (Oi_{cord} cells are used because of the extreme rarity of Oi_{adult} cells). The tests at 30°C should also include the albumin displacement technique with OI, Oi and patient's cells as it has been shown that activity in albumin at this temperature is a reasonable indicator that a cold antibody is of clinical significance.²³ Care should be taken when reading these tests to maintain the microscope slides at 30°C.

The majority of patients suffering from cold haemagglutinin disease (CHAD) have auto anti-I in their serum, although some have auto anti-i or an antibody showing no obvious specificity. Anti-I and anti-i may be associated with *Mycoplasma pneumoniae* and infectious mononucleosis, respectively. Rarely, anti-Pr is found; this reacts equally well with OI and Oi cells, but weakly or not at all with the papainised cells as the Pr antigen is destroyed by proteolytic enzymes.

HAEMOLYSIN TESTS

The potential for autoagglutinins (usually cold reacting ones) to cause complement activation and intravascular haemolysis *in vivo* is assessed *in vitro* by performing haemolysin tests. As previously mentioned, collection of samples and separation of serum for these tests must be carried out strictly at 37°C, otherwise autoabsorption of antibody may occur—this is particularly important in patients suspected of having paroxysmal cold haemoglobinuria where the levels of autoantibody may be low to start with.

Method—As the patient's serum is likely to be complement deficient, complement must be added to the tests by preparing doubling dilutions (from 1 in 2 to 1 in 16) of patient's serum in pooled group O serum less than 24 hours old; 10 drops of each dilution are placed in two rows of 10 × 75 mm tubes. As better correlation with the *in vivo* effect of the

antibody is given if the serum is first acidified to a pH of about 6.8, one drop of 0.2 M HCl is carefully added to each tube. The acidified serum dilutions and a 50% suspension of pooled group O red cells (made up in the complement rich serum) are prewarmed to 37°C, one drop of the red cell suspension is added to each tube and after mixing, one row is removed and incubated at 18°C for two hours, the other row remaining at 37°C. The red cells are resuspended, centrifuged and the supernatants examined for haemolysis. It is important to compare the colour of the test supernatant with that of the original serum dilution, particularly if the latter already shows evidence of lysis. The acid haemolysis tests at 18°C are usually more strongly positive than the ones at 37°C.

Haemolysins are infrequent in warm type autoimmune haemolytic anaemia. They may very rarely cause severe intravascular haemolysis, but usually they have minimal clinical effects and are only detected using papainised cells.^{15 24}

Method—Three drops of a 5% suspension of prewarmed, papainised pooled O cells are added to three drops of prewarmed serum dilutions (prepared as above but not acidified), mixed and incubated at 37°C for one to two hours before centrifugation and examination of the supernatants.

In patients with unexplained haemolysis or in those with suspected paroxysmal cold haemoglobinuria an indirect Donath–Landsteiner test²⁵ is performed.

Method—Doubling dilutions of the patient's serum (from 1 in 2 to 1 in 16) and a 50% suspension of pooled O cells are prepared in fresh pooled O serum as before. Ten drops of each dilution are placed in pairs of 10 × 75 mm tubes set in two rows and prewarmed to 37°C; one drop of the red cells is then added and mixed. One set of dilutions is left at 37°C for two hours while the other is placed in melting ice (0°C) for one hour before being transferred back to 37°C for a further hour; the contents of the tubes are then mixed and centrifuged. A positive result is indicated by lysis in the tests that have been cooled and re-warmed, with no lysis in the tests which remained at 37°C throughout. Classically, the Donath–Landsteiner antibody shows P blood group specificity and, if possible, this should be confirmed by repeating the test using pp cells and obtaining a negative result.

If paroxysmal cold haemoglobinuria is suspected but the indirect Donath–Landsteiner test is negative, further investigations are carried out. Repeating the test using papainised red cells sometimes gives a positive result in cases where the level of autoantibody is particularly low. A two-stage procedure in which the complement is added after the tests have been at 0°C for one hour overcomes the problem of inhibition of the autoantibody by globoside present in the fresh serum used as a source of complement.

ABSORPTION OF AUTOANTIBODIES

Strongly reacting serum autoantibodies may mask the presence of alloantibodies and thus put the patient at risk of a transfusion reaction. The recent suggestion that the risk of allo-immunisation is generally overstated²⁶ was quickly refuted²⁷ and in our experience allo-antibodies are found in about 14% of patients with autoantibodies.²⁸ Both cold and warm autoantibodies may hamper the detection of alloantibodies, especially when using indirect antiglobulin techniques, and several methods for absorbing autoantibodies are available to overcome this difficulty.

Cold autoagglutinins can be either auto-absorbed or in the case of anti-I (but not anti-i or anti-Pr) absorbed using rabbit erythrocyte stroma (Organon Teknika, Boxtel, The Netherlands). Rabbit red cells possess structures similar to the human I, H and HI antigens and the stroma will remove these cold agglutinins but not most clinically significant allo-antibodies. (Note that anti-B and anti-P₁ are also removed by the stroma, and the absorbed serum should therefore not be used for ABO grouping or for routine compatibility testing.)

Method for autoabsorption—One volume of packed patient's red cells (which have been washed four times in PBS prewarmed to 37°C) is added to one volume of patient's serum. The cells and serum are mixed and placed at 4°C for at least one hour (or overnight if time allows) and centrifuged; the serum is now ready for testing. Further absorptions may be carried out if necessary.

Method for absorption using rabbit erythrocyte stroma—Patient serum (1 ml) is added to a tube of stroma (from which the preservative has been removed), mixed well, placed at 4°C for a minimum of 30 minutes (or for 60 minutes if time allows) and centrifuged. The absorbed serum is removed for testing or for further absorption if required.

Warm autoantibodies can be removed from a patient's serum using either differential or autoabsorption techniques. If sufficient patient red cells are available and the patient has not been transfused within the last four months, then absorption with autologous red cells treated with dithiothreitol and papain (ZZAP) may be used.²⁹ The dithiothreitol removes some, if not all, of the autoantibody already bound to the red cells and the papain, by exposing further antigen sites, renders the cell more efficient at absorbing further antibody.

Method for autoabsorption—To absorb 0.5 ml of patient's serum, at least 1 ml of packed, treated autologous red cells is required; as some of the cells lyse during the dithiothreitol/papain treatment, it is advisable to process at least 1.5 ml of red cells. The patient's red cells are washed three times in PBS (pH 7.0), packed, mixed with two volumes of dithiothreitol/papain working solution (2.5 ml 0.2 M dithio-

threitol plus 0.5 ml 1% papain solution plus 2 ml PBS (pH 7.0)), incubated at 37°C for 30 minutes, washed three times (removing as much saline as possible after the last wash) and divided into two or three equal volumes. One volume of patient's serum is added to one aliquot of packed cells, mixed well, incubated at 37°C for 30 minutes, centrifuged at high speed, and transferred to the second aliquot of red cells, mixed well and incubated at 37°C as before, repeating the absorption for a third time if sufficient cells are available. The absorbed serum is tested against a comprehensive panel of fully typed red cells using a LISS indirect antiglobulin technique.

In practice, patients with strong autoantibodies that require absorption are often anaemic and, because of the requirements for grouping, direct antiglobulin testing and elution studies, there are usually insufficient red cells available for successful autoabsorption even when several anticoagulated blood samples have been collected. In addition, transfusion within the previous four months can mean that the circulating transfused red cells may complicate the procedure by regaining the potential to absorb weak alloantibodies after treatment with dithiothreitol and papain. For these reasons, we tend to carry out differential absorptions in preference to autoabsorption.

Differential absorption uses carefully selected group O red cells to identify or exclude the presence of alloantibodies in the Rh, Kell, Duffy, Kidd, Lewis, and MNS systems. Blood of suitable type can be aliquoted into vials and stored at 4°C for up to three weeks, giving sufficient red cells for multiple tests. Examples of two cell types suitable for absorption studies are Orr, kk, Jk^{a+b-} and O R₁R₁, kk, Jk^{a-b+}, one of them also being Le^{a-b-} if possible; the Duffy and MNS antigens are not important as they are destroyed by the enzyme treatment used in the procedure and would therefore not absorb the corresponding alloantibodies. (Note that anti-Lewis antibodies should already have been detected in the 18°C saline agglutination panel.) The potential disadvantage of differential absorption is the possibility that an antibody to a high incidence antigen may be removed by the absorbing cells, but in practice such antibodies are so rare that this is not a major problem.

Method for differential absorption—Approximately 3–4 ml of each of the absorbing cells is washed three times in PBS (pH 7.0) and an equal volume of 1% papain solution is added. After incubation at 37°C for 15 minutes, the cells are washed a further three times (ensuring that they are well packed and all saline is removed after the final wash) and separated into three or four aliquots. To the first aliquot of each absorbing cell, an equal volume of patient's serum is added, mixed, incubated at 37°C for 15–30 minutes and centrifuged. The serum is then transferred to another aliquot of the same absorbing cell and the procedure repeated. In most cases, re-

peating the procedure up to four times is adequate to remove strong autoantibodies and permit the detection of concomitant alloantibodies. The two absorbed serum samples are tested against a comprehensive panel of red cells using a LISS indirect antiglobulin technique.

ESTIMATION OF SERUM HAPTOGLOBINS AND SERUM PROTEIN ELECTROPHORESIS

Haptoglobins are α -2-glycoproteins which have the property of combining with haemoglobin; the normal serum range is 0.4–2.0 g/l. Their concentration is regarded as a reasonably sensitive indicator of haemolysis, although in conditions where there is an increased rate of synthesis (for example, inflammatory disease, malignancy and steroid administration) greater rates of red cell destruction may be necessary to depress the concentration, and lower concentrations may be found normally during pregnancy. If a patient's serum haptoglobin concentration is <0.1 g/l, then increased red cell breakdown is occurring, but this is not necessarily because of autoimmune haemolysis. For example, if other tests do not support an immune aetiology, it may be advisable to perform Ham's test in case the patient is suffering from paroxysmal nocturnal haemoglobinuria.

Serum protein electrophoresis is sometimes helpful in the investigation of patients with suspected autoimmune haemolysis by revealing general protein abnormalities. Paraproteins or a general increase in γ -globulins can give rise to rouleaux formation (which can be confused with agglutination) and to positive direct antiglobulin tests due to non-specific adsorption.

Supplying blood for transfusion

Blood to be given to patients suffering from autoimmune haemolytic anaemia should have the same ABO group as the potential recipient, except in the rare instances where the autoantibodies show specificity for the A or B antigens; in these cases, group O blood is preferred.²⁸ To avoid stimulating the production of Rh alloantibodies and to prevent transfusion reactions from such antibodies already present but masked by the autoantibodies, the blood to be transfused should have the same Rh antigens as the patient (table). It is preferable to select blood using this principle even if the patient's autoantibody shows specificity within the Rh system.²⁸ The only exception would be if the haemolysis was fulminating and the specificity simple, in which case it might be considered that the advantage of possible increased red cell survival would outweigh the potential for stimulating alloantibody production. In our experience such circumstances are exceedingly rare. As 99.8% of the population possess the k antigen (91% being kk), Kell negative blood (that is, kk) should also be routinely chosen to avoid stimulating the development of anti-Kell (and to prevent a reaction if it is present but masked).

Rh genotypes of blood usually selected for patients with red cell autoantibodies

Patient's Rh genotype	Rh genotype of suitable blood
R ₁ r	R ₁ R ₁ , R ₁ r or rr
R ₁ R ₁	R ₁ R ₁
R ₁ R ₂	Any Rh genotype
rr	rr
R ₂ r	R ₂ R ₂ , R ₂ r or rr
R ₂ R ₂	R ₂ R ₂

The blood selected should, of course, not possess antigens to any other alloantibodies present. The most important non-Rh alloantibodies to identify are those in the Duffy, Kidd, Lewis, and MNS (and Kell) systems.

The units of blood chosen are cross-matched against the patient's serum using saline agglutination methods at 30°C (to avoid problems with clinically insignificant cold antibodies) and saline, albumin, papain, and LISS indirect antiglobulin techniques at 37°C. In cases where absorption procedures have identified low incidence antibodies, cross-matching is also carried out with the absorbed serum. Red cell units which have been concentrated with the addition of "saline, adenine, glucose-mannitol" (that is, SAG-M cells) are given by choice.²⁸ If these are not available, then concentrated red cells are suitable. Previously, it was thought necessary to wash the cells to remove plasma (which may be rich in complement) before transfusing patients with demonstrable haemolysins—for example, those with chronic cold haemagglutinin disease or with the Donath–Landsteiner antibody. Our experience has shown that this is unnecessary if SAG-M units are selected.²⁸

The blood is almost always incompatible by at least one technique and these units are therefore issued to the patient with a label stating "not compatible but considered suitable for".²⁸ In the rare cases where absorption of the autoantibody is unsuccessful, or where insufficient time is available to rule out the presence of masked alloantibodies, a note is issued with the blood warning the clinician of the increased risk of an adverse reaction and advising that the patient is kept under close supervision during the transfusion. If strong cold autoagglutinins are present in the patient's serum, labels stating the advisability of transfusing through a warmer are attached to the blood packs.

No critically ill patient with autoimmune haemolytic anaemia should die through lack of blood and in extreme circumstances we would empirically transfuse group Orr Kell negative SAG-M blood, possibly also giving steroids or intravenous immunoglobulin,³⁰ or both, to prevent or reduce possible untoward consequences. The use of monoclonal antisera, the LISS antiglobulin technique and rapid absorption procedures allow suitable blood to be selected and cross-matched in under two hours, a fraction of the time taken only a few years ago. Since January 1983, we have issued more than 21 500 units of blood to over 5000 recipients with red cell autoantibodies without, as far as we are aware, any serious ill effects.

Investigation of drug induced immune haemolytic anaemia

Finally, many drugs and chemicals can bring about red cell destruction *in vivo* by an immune mechanism and although many cases are not autoimmune in nature, it is convenient to outline briefly the three main types (drug adsorption, immune complex and autoimmune) and their investigation in this broadsheet. Drug induced immune haemolysis is very complex and for an in-depth consideration of the subject the reader is recommended to consult one of the excellent recent review articles.^{31–34} Even though such haemolysis is rare, occurring in about one per million of the population,³³ it may cause significant problems clinically.

The drug adsorption (or hapten) type is usually associated with large doses of medication. The drug (for example, penicillin, cephalosporin or carbimazole) coats the patient's red cells and leads to the development of IgG class antibodies against a combination of drug and red cell membrane, resulting in the red cells being destroyed by the mononuclear phagocyte system. The immune complex type of immune haemolysis can occur when small doses of drug (for example, quinine, rifampicin and tolbutamide) are being taken. Antibodies are produced against the drug and form immune complexes which attach to the red cells and activate complement; the resulting intravascular haemolysis may be severe and lead to renal failure or may even be fatal.

The serological investigation of both these types of drug induced haemolysis is fraught with difficulties. For example, certain drugs do not readily dissolve in aqueous solutions so that preparing an isotonic medium can be difficult. This can be further complicated by the presence of inert filler in some tablets and capsules making control of the amount of drug in solution almost impossible. Other problems include the likelihood that the antibodies are directed against metabolites of the drug³² or that the patient may be taking several different medicines. Consulting the established literature is invaluable in deciding the approach to the investigation. When a solution of drug has been prepared, an attempt is made to coat it onto group O red cells, initially by incubation of drug solution and red cells at 37°C. If this fails, it may be necessary to vary the pH or use cross-linking techniques. The coated cells are then used in serological tests (with and without complement) utilising the patient's serum and a red cell eluate; lysis and agglutination reactions in saline and with indirect antiglobulin tests (using monospecific anti-IgG and anti-C3 reagents) are noted. Positive results with drug coated cells but negative reactions with uncoated red cells indicate the drug adsorption type of immune haemolysis. An immune complex mechanism is suggested if positive results are obtained with untreated group O red cells and mixtures of drug solution and patient's serum. The direct antiglobulin tests often show complement coating the red cells, but various classes of immunoglobulin can sometimes be detected, particularly if sensitive enzyme linked methods are used.

There are no laboratory tests to identify the third type of drug induced immune haemolysis. Patients treated with methyl dopa—for example, can develop autoantibodies indistinguishable from those found in warm type autoimmune haemolytic anaemia. In most cases there is little or no clinical effect, but in others overt haemolysis develops. It has been postulated that the drugs affect T suppressor lymphocyte control of B lymphocytes, permitting the production of autoantibodies. Methyl dopa is prescribed less frequently nowadays, and these cases are seen less often, whereas a few years ago methyl dopa accounted for some two thirds of all patients with a drug induced haemolysis.³⁵ Currently, most cases of the autoimmune type of drug haemolysis are caused by levodopa or non-steroidal anti-inflammatory agents (for example, ibuprofen, mefenamic acid, naproxen).

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