

X. Identification of Unexpected Alloantibodies

A. Introduction

1. ***Unexpected alloantibodies are antibodies other than naturally occurring anti-A or -B.***
2. Such antibodies are found in some 0.3-2% of the population, depending upon the group of patients or donor studied and the sensitivity of the test methods.
3. *Immunization to foreign RBC antigens may result from:*
 - a. pregnancy
 - b. transfusion
 - c. following deliberate injection with immunogenic material
 - d. In some instances the immunizing event is unknown.
4. Once an unexpected antibody is detected in prenatal or pretransfusion testing, **its specificity should be determined and its clinical significance assessed.**
5. ***A clinically significant antibody is one that:***
 - a. Shortens the anticipated survival of transfused RBCs
 - b. Has been associated with HDN.
6. **The clinical significance varies.**
 - a. Some antibodies cause destruction of incompatible RBCs within a few hours or even minutes.
 - b. Others decrease the anticipated survival by only a few days.
 - c. Documented experience with other examples of the same antibody specificity can be used in assessing the relative clinical significance of an antibody.
7. Unfortunately, for some antibodies there are no data and *decisions must be based on the premise that an antibody is not clinically significant unless active in vitro at 37 C and/or by the IAT.*
8. Determining the specificity of antibodies encountered in pretransfusion testing is important in assessing the need to select antigen negative blood for transfusion.
9. Patients with **clinically significant** antibodies should receive blood that has been found to lack the corresponding antigen using potent antisera.
10. In **prenatal** testing, knowing the specificity and immunoglobulin class of an antibody helps predict the likelihood of HDN.
11. While it is not crucial to identify unexpected antibodies in donor bloods, such testing is often undertaken for the procurement of reagent antisera or teaching samples.

B. General Procedures

1. An adequate quantity of test serum and RBCs is essential to the resolution of any serological problem. *Either serum or plasma may be used.*

2. An EDTA-anticoagulated blood sample is preferred for studies of autologous RBCs to avoid problems associated with the in vitro uptake of complement components by RBCs that occurs when clotted blood samples are used.
3. Medical History.
 - a. *It is useful to know a patient's clinical diagnosis, number of pregnancies, transfusion history and drug therapy.*
 - b. A recent transfusion may necessitate the use of procedures such as red cell separation techniques to determine the blood type of the autologous red cells.
4. It is appropriate to test the serum under investigation at all test phases at which antibody activity was initially detected.
 - a. Additional antibodies may become apparent at different test phases.
 - b. *Reactivity of some antibodies may be increased by **extending the incubation time, lowering temperatures, increasing the serum to cell ratio or by using sensitive methods such as enzyme (ficin) techniques.***
5. Advantages of using *enzyme techniques* are:
 - a. *Enhances reactivity of Rh antibodies* and complement binding examples of anti-Lea and anti-Jk^a.
 - b. *Denatures some blood group antigens, especially M, N, S, and Fy*, so if multiple antibodies are present and one has specificity for MNS or Fy, it aids in identification of other antibodies.
6. The serum under investigation should be tested by the desired techniques with a panel of eight or more *group O reagent RBC* samples of *known blood group phenotype*.
 - a. To be functional, a reagent RBC panel must make it possible to identify with confidence the most frequently encountered, clinically significant alloantibodies.
 - b. A distinct pattern of reactivity should be apparent for most examples of single alloantibodies.
 - c. There must be sufficient RBC samples that lack, and sufficient RBC samples that carry, the antigens that individuals frequently make antibodies to.
7. It is important to know how the serum under investigation reacts with the autologous RBCs (auto control) to determine whether allo-antibody, auto-antibody or both are present in the serum.

C. Considerations in Interpreting Serological Results

1. *Alloantibodies of some blood group specificities **frequently** display consistent serologic characteristics, it is important to look for characteristics such as:*
 - a. What are the effects of temperature, suspending medium or enzymes on the reaction with a particular cell sample.
 - b. Is there any variation in the strength of agglutination observed among reactive RBC samples.
 - c. Is hemolysis present.
 - d. Is the auto-control positive or negative.

2. Single alloantibodies.
 - a. It is *usually easy to recognize* the specificity of a single allo-antibody that yields clear-cut positive and negative reactions with reagent RBC samples.
 - b. While the test phase at which a serum reacts is suggestive of specificity, it is important to remember that exceptional examples will be encountered.
 - c. The *strength of observed reactions may vary due to dosage affect, variation in the amount of antigen on the cell, deterioration of the antigen* during storage or the presence of multiple antibodies.
 - d. Although a serum displays a reaction pattern indicating a single antibody, **it is important to remember that additional antibodies may be present.**
3. Special considerations with Rh antibodies.
 - a. *If anti-E is identified in the serum of a transfusion candidate, the additional presence of anti-c should be considered.*
 - b. Determine the Rh phenotype of the patient. *Even when anti-c is not detectable, it is advisable to select c-, E- (R_1R_1) blood for transfusion to R_1R_1 patients with anti-E since anti-c is a **common cause of delayed HTR.***
 - c. The reverse situation causes less of a problem. If anti-c is identified the additional presence of anti-E may not be determined unless rare RzR1 RBCs are used. Also, almost all c- donor units will be E-.
4. Phenotype of autologous red cells.
 - a. *Once an allo-antibody has been identified in a serum it is necessary to test the patient RBCs for the corresponding antigen.*
 - b. When an allo-antibody is present in the serum, the corresponding antigen will be absent on the autologous RBCs.
 - 1) When the patient types negative for the antigen to which the antibody is directed, this provides additional confirmation of antibody specificity.
 - 2) If the patient types positive for the antigen, this indicates misinterpretation of the antibody work up.
 - c. *Antigen typing **cannot** be performed on blood samples of recently transfused individuals unless it is done on a pretransfusion sample **or** a cell separation technique is performed (separating patient RBCs from transfused RBCs).*
5. Probability
 - a. For conclusive antibody identification, there must be sufficient reagent RBC samples tested that lack, and sufficient that carry, the antigen to which an antibody appears to display specificity.
 - b. ***There must be a minimum of three cells which possess the antigen that react and three cells which lack the antigen which do not react.***

- c. It is important to remember the limitations when a patient has an antibody against a moderately high incidence antigen such as e. It is necessary to test additional e- RBC samples before conclusively assigning specificity, and of equal importance, identify possible "hidden" or "masked" antibodies.

D. Multiple antibodies.

1. When a serum contains two or more alloantibodies, it may be difficult to interpret the results of serum studies using a single panel of reagent RBCs.
2. ***Multiple alloantibodies usually present in one or more of the following ways:***
 - a. The observed pattern of reactive and nonreactive tests does not fit that of a single antibody.
 - b. Reactions of variable strength are observed with the reactive RBC samples that cannot be explained on the basis of dosage.
 - c. Different RBC samples react at different test phases.
 - d. Unexpected reactions are obtained when attempts are made to confirm the specificity of a suspected single antibody. For example, if a serum suspected of containing anti-e is found to react with additional e- RBC samples, it is possible that either another antibody is present, **or** the suspected antibody is not really anti-e. In such a situation additional e- RBC samples must be tested.

E. Antibodies to high incidence antigens.

1. *An allo-antibody to a high incidence antigen should be suspected when all reagent RBC samples are reactive, **but the auto control is nonreactive.***
2. Once multiple antibodies are ruled out these problems are often referred to an immunohematology reference laboratory, which have the necessary rare cells for performing such workups.
3. *The patient's siblings are often the best source* of serologically compatible blood for patients with antibodies to high incidence antigens.

F. Antibodies to low incidence antigens.

1. When a serum sample reacts only with RBCs from a single donor unit the most likely possibilities to consider are: the unit is ABO incompatible, the donor cells have a positive DAT or are polyagglutinable.
2. Reactions between a serum and a single donor or reagent RBC sample may also be caused by antibodies to low incidence antigens.
3. If RBCs known to carry low incidence antigens are available, the serum may be tested against them.
4. It is inappropriate to delay transfusion while such studies are undertaken, since finding compatible blood will not be a problem.

G. Anomalous serological reactions.

1. Antibodies to a variety of drugs and additives can cause positive results in antibody detection and identification tests.

2. There are many times that reactions will be obtained, and attempts to increase the strength of reactivity are unsuccessful.
 - a. After every attempt to identify the specificity are unsuccessful, an interpretation of “*unable to determine antibody specificity*” is made.
 - b. *The patient must be transfused with serologically (crossmatch) compatible blood.*

H. Selected Serological Procedures.

1. When a pattern of reactions fails to indicate specificity, or when the presence of an antibody is suspected but cannot be demonstrated, use of the following procedures may be helpful.
2. **Enzyme techniques** are very useful in antibody identification studies.
 - a. Treatment of RBCs with proteolytic enzymes enhances the reactivity of Rh, P, I, Lewis and complement binding alloantibodies such as anti-Jka.
 - b. Antigens of M, N, S and Fy are depressed or destroyed.
 - c. Enzyme techniques should be used whenever a weakly reactive antibody may be an Rh antibody or when a patient has multiple antibodies and one of them is a Fy.
3. **Temperature reduction** is useful for alloantibodies (e.g., anti-M, -P1) that react better at cold temperatures. Specificity may become apparent at or below 22 C.
 - a. An auto-control is especially important for tests at cold temperatures, because many sera contain cold reactive auto-antibodies.
 - b. Anti-I specificity is confirmed by testing the patient serum with adult (I+) and cord (i+) cells at 4 C. Positives with adult cells and negative with cord cells confirms anti-I specificity.
4. **Increasing the serum to cell ratio** increases the amount of antibody in the test system which may increase the strength of reactivity of antibodies present in low concentrations.
 - a. Increasing to four drops is recommended, but an increase of 5-10 drops may be indicated and extending incubation time up to 60 minutes, mixing the solution periodically.
 - b. It is important to remove the serum prior to washing, as 3 washes would not adequately remove this volume of serum.
 - c. **NOTE:** Be cautious when attempting to increase the serum to cell ratio in LISS tests that require equal volumes of serum and cells.
5. **Increasing incubation time** to 30-60 minutes may improve reactivity and help clarify the observed pattern of reactions.
6. **Decreasing the pH of the reaction medium** to 6.5 by the addition of 0.2 N HCL to the patient serum enhances the reactivity of certain antibodies, most notable, some examples of anti-M.

7. Some blood group antibodies react preferentially in test systems utilizing **Low Ionic Strength Salt (LISS)** solutions.
 - a. LISS reagent accelerate antibody uptake (IE, the first state of the hemagglutination reaction that involves association of antibody molecules to RBCs).
 - b. A variety of LISS procedures have been described.
8. **Use of thiol reagents** such as dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) cleave inter-subunit disulfide bonds of **IgM** molecules.
 - a. The IgG molecules are relatively resistant to such cleavage, so treatment results in destruction of IgM but leaves IgG intact able to react in-vitro.
 - b. *The applications of DTT and 2-ME in immunohematology include:*
 - 1) Determining the immunoglobulin class of an antibody, especially if the antibody has the potential of causing HDN.
 - 2) Dissociating RBC agglutinates caused by IgM antibodies (e.g., spontaneous agglutination of RBCs caused by potent cold reactive auto-antibodies).
 - 3) Identifying specificities in a mixture of IgM and IgG antibodies, particularly when an agglutinating IgM antibody masks the presence of IgG antibodies.
9. **Prewarmed Technique**
 - a. Sometimes cold auto-agglutinins may demonstrate high thermal amplitude resulting in false positive reactions at 37C and AHG.
 - b. *After confirmation of the cold agglutinin* it is acceptable to perform a prewarmed antibody screen and crossmatch:
 - 1) Place RBCs to be tested in appropriate test tubes and place in 37C heat block.
 - 2) Prewarm tube of serum to 37C.
 - 3) Add 3 to 4 drops of prewarmed serum to prewarmed cells and incubate 1 hour.
 - 4) Without removing tubes from heat block add saline that has been prewarmed to 37C.
 - 5) Immediately spin and wash 2 additional times with the warm saline.
 - 6) Perform the AHG procedure.
 - c. The prewarmed technique can confirm that only a cold auto-agglutinin is present or detect the presence of clinically significant underlying alloantibodies.

I. Inhibition Tests

1. *Some blood group antigens exist in soluble form in such body fluids as saliva, urine or plasma.* These substances are useful in antibody identification studies, either to confirm antibody specificity by inhibition or to neutralize antibodies that mask the presence of concomitant non-neutralizable antibodies. The following soluble blood group substances can be used in antibody identification tests.

2. *Lewis substances.*

- a. Le^a and Le^b substances are present in the saliva of persons with the appropriate Lewis phenotype, and Lewis substance can be prepared from saliva. Most blood banks use commercially prepared Lewis substance.
- b. Lewis substance will neutralize Lewis antibodies in a patient specimen, allowing the detection of underlying, clinically significant alloantibodies.

3. *P¹ substance*

- a. Soluble P¹ substance is present in hydatid cyst fluid as well as pigeon eggs.
- b. P¹ may mask the presence of underlying alloantibodies. The addition of P¹ substance to the patient's serum causes neutralization of the anti-P¹, allowing the detection of underlying alloantibodies.

4. *Sd^a (Sid) substance*

- a. Sd^a blood group substance is present in soluble form in various body fluids, with the most abundant source being urine.
- b. The urine is added to the patient serum, causing neutralization of the anti-Sd^a.
- c. Aids in the detection of underlying alloantibodies.

J. Titration

1. The titer of an antibody is usually determined by testing serial two-fold dilutions of the serum against selected RBC samples.
2. Results are expressed as the reciprocal of the highest serum dilution that causes macroscopic agglutination.
3. *Prenatal studies.*
 - a. When the antibody is of a specificity known to cause HDN or when the clinical significance of the antibody is unknown, the results of titration studies, outcome of previous pregnancies are used to assess the need for amniocentesis.
 - b. Rising titers are indicative of active immunization of the mother.
4. *Antibody identification*
 - a. Some antibodies cause agglutination of virtually all reagent RBC samples, but specificity is indicated by differences in the strength of reactivity with each sample in titration studies.
 - b. The titration procedure is not used very commonly for this purpose.
5. *HTLA antibodies.*
 - a. HTLA antibodies react very weakly in the undiluted state but, unlike most weakly reactive antibodies (e.g., anti-D with a titer of 4), react at a high dilutions (e.g., 1 in 2000).
 - b. Such antibodies include anti-Ch, -Rg, -Cs, -Yk, -Kn^a, -McC^a and -JMH.

- c. When weak reactions are observed in the IAT, titration studies may be used to establish whether or not the reactions are due to the presence of an HTLA antibody.

K. Adsorption

1. *Antibody can be removed from a serum by adsorption to RBCs carrying the corresponding antigen.*
 - a. The antibody forms a complex with RBC membrane-bound antigens.
 - b. When the serum and RBCs are separated, the antibody remains attached to the RBCs.
 - c. Subsequent elution of the bound antibody can often give additional useful information.
2. *Adsorption techniques are useful in situations such as:*
 - a. Removing auto-antibody activity to permit detection of coexisting alloantibodies.
 - b. Removing unwanted antibody from a serum that contains an antibody suitable for reagent use.
 - c. Confirming the presence of antigens on RBCs through their ability to remove a specific serum antibody.
 - d. Confirming the specificity of an antibody by showing that it can be absorbed only to RBCs of a particular blood group phenotype.
 - e. Separating multiple antibodies present in a single serum sample.

L. Elution

1. *Elution techniques free antibody molecules from sensitized RBCs so the recovered antibody can be tested.*
2. A variety of methods are employed with the primary objective being breaking the bond between the antigen and the antibody.
3. *Elution techniques are primarily used for:*
 - a. Identification of an antibody coating a baby's RBCs in the case of HDN.
 - b. Identification of an antibody causing an acute or delayed hemolytic transfusion reaction.
 - c. Investigation of a positive DAT.
 - d. Concentration and purification of antibodies, the detection of weakly expressed antigens and the identification of multiple antibodies.
 - e. Preparation of antibody-free intact RBCs for use in phenotyping or autoabsorption.
4. Technical factors which influence the success of the elution techniques include:
 - a. Incorrect technique.

b. Incomplete washing

- 1) If cells are incompletely washed contaminating serum antibody will cause false positive reactions.
- 2) An aliquot of saline from the last wash is saved and tested in parallel. *Positive reactions with the last wash invalidates the test.*

c. Binding of proteins to glass surfaces.

d. Dissociation of antibody before elution.

e. Instability of eluates.

M. Selection of Blood for Transfusion After Antibody Identification

1. After antibody identification studies are complete *determine the clinical significance of the antibody.*
2. *If the antibody is clinically significant antigen negative donors must be found and crossmatched for the patient, a Coomb's crossmatch must be done.*
3. If the antibody is *not clinically significant* it is not necessary to provide antigen negative blood, but the *donors must be compatible by the Coomb's crossmatch.*