e-GRIPS Online Continuing Education

Summary: Introduction to Antibody Identification

Objectives

After completing the learning module, the learner will be able to:

1. Discuss the limitations of antibody detection and identification.
2. List the basic steps of antibody identification.
3. Interpret antibody reactivity patterns for single antibodies, multiple antibodies, and autoantibodies, as well as reactions relating to recent transfusions.
4. Discuss special techniques used in the enhancement, detection and identification of antibodies.
5. Compare and contrast the autocontrol and DAT.
6. Explain the "ruling out" or exclusion process.
7. Discuss how probability (p) is used in antibody identification.

Patient Sample Requirements: Examples

- Patient name
- Medical record number
- Social Security Number
- Date of birth
- External identification system number

Serum or Plasma?

Plasma	Serum
Not suitable for detecting complement-activating antibodies	Complement can bind to cells during storage
Preferred for some testing methodologies	Aggregates can form in fibrin clots from incompletely clotted samples which can be mistaken for agglutination

Reagent Red Cells

All antibody detection methodologies require the use of reagent red cells to provide antigens that allow sensitization within the test system if a patient antibody is present, and use of AHG reagent that allows for agglutination of incomplete antibodies. Enhancement media is optional but provides increased sensitivity when used.

Reagent red cells used in antibody screening tests are available in two-, three-, or even four-cell sets. All screening cells are group O to avoid interference from ABO antibodies. Each vial within a set is from a single donor. Pooled screen cells may be used for testing blood donors, but not with recipients. Use of a two-, three-, or four-cell set is up to each facility. Within the set, the D, C, c, E, e, K, k, Fy^a, Fy^b, Jk^a, Jk^b, Le^a, Le^b, P_1, M, N, S, and s antigens must be expressed.

Some weakly reactive antibodies react only with screening cells that have double-dose antigens. This phenomenon is termed dosage, and is most commonly demonstrated by antibodies in the Rh, Duffy, MNS, and Kidd systems. It is recommended that there be double-dose expression of some antigens within the screening cell set to allow for detection of antibodies that show dosage.

Enhancement Media

Enhancement media are added to the antibody test system to increase the speed and sensitivity of antibody attachment to red blood cell antigens. Enhancement media currently available are low-ionic strength solutions (LISS), bovine serum albumin (BSA), polyethylene glycol (PEG), and proteolytic enzymes.

LISS additive solutions can contain glycine in an albumin solution, and work by reducing the ionic strength of the test system. Each LISS manufacturer uses different formulations, so the manufacturer's package insert should be checked for the specific ingredients. Decreasing the ionic strength helps to lower the zeta potential, which helps to increase the uptake of antibody during the sensitization phase. LISS is economical and offers good test system sensitivity, but it may also enhance cold autoantibodies. It is important to follow the manufacturer's instructions when using any serological reagents. For example, using increased amounts of serum to enhance weakly reactive antibodies is not recommended with LISS because altering the serum to cell ratio changes the ionic strength of the test system, thus decreasing sensitivity. It has also been reported that some weakly reactive examples of anti-K may be missed in LISS testing systems.

BSA is prepared from bovine serum in a 22% or 30% solution. BSA influences the second stage of agglutination (lattice formation) by reducing the zeta potential. This disperses the charges in the test system, and allows red cells to approach each other, increasing the chances of agglutination. The exact effect of BSA is not clearly understood, but it may affect the degree of water hydration of the RBC membrane itself. BSA works well in enhancing Rh-system antibodies, but is not sensitive enough to dependably detect antibodies to other blood group systems. BSA also requires increased incubation time for optimal results.

PEG in a LISS solution works by removing water from the test system, which concentrates any antibodies present. The LISS environment also works to enhance antibody uptake. PEG offers increased sensitivity over LISS and BSA, while discouraging the enhancement of IgM (cold) antibodies, which do not react well or not at all in PEG systems. PEG can cause nonspecific aggregation of red cells; therefore, the 37 C reading is not recommended. PEG can enhance warm autoantibodies, and is not appropriate for use in patients with elevated protein levels as protein precipitation can occur.

Enzymes are not usually used as a potentiator, but are used as a tool for complex antibody problems. Enzymes destroy some antigens (M, N, S, Fy^a, Fy^b), and enhance antigen-antibody reactions in other blood group systems (Rh, P, I, Kidd, Lewis). Cold and warm autoantibodies are enhanced when enzymes are used. The use of enzymes should never be the sole methodology, but can be very useful in antibody identification involving multiple antibodies.

There is no one perfect method for antibody detection and identification testing, but by having knowledge of how different enhancement media work; you can choose the best method for each particular antibody case.

Antihuman Globulin (AHG) Reagent

AHG reagent allows for the agglutination of incomplete antibodies, or those that are not effective at causing visible agglutination in a saline test system. AHG reagent must contain anti-IgG when used for antibody detection and pretransfusion compatibility testing. Polyspecific AHG contains both anti-IgG and anti-C3d. The presence of anti-complement activity in polyspecific AHG reagent may lead to the detection of clinically insignificant antibodies. Monospecific AHG reagent containing only anti-IgG is preferred by most workers to avoid unwanted reactivity due to *in-vitro* complement binding by cold-reactive antibodies.

IgG-Sensitized Red Cells

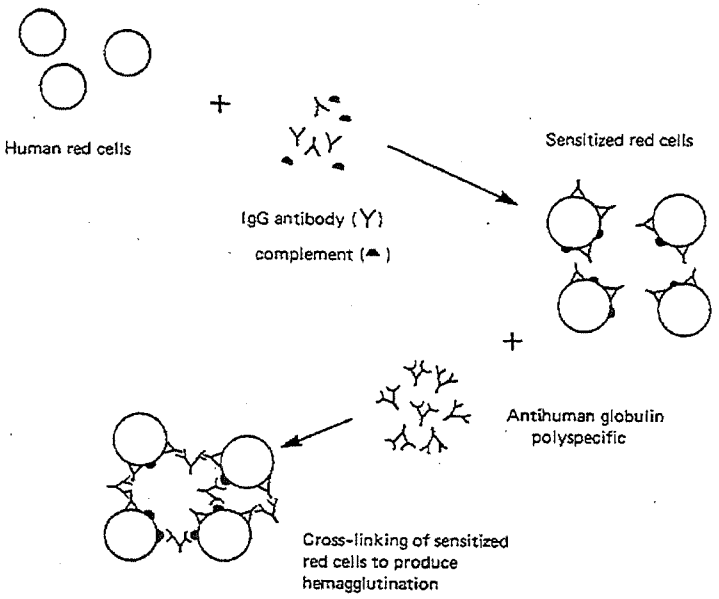
Coombs Control Cells (CCC), or "check cells" (CC) are IgG-coated red cells prepared by coating Rh-positive cells with anti-D. AABB *Standards* require CCC to control negative AHG tests in antibody detection and crossmatching procedures. CCC must react when added to negative AHG tests or the test must be repeated. Using CCC verifies adequate washing was performed and AHG was added and was

working properly. Complement coated red cells are used to control tests using antisera directed against complement components.

Antibody Screening Test

No matter what testing methodology is used to perform the antibody screen, the patient's serum or plasma is incubated with screening cells, and an indirect antiglobulin test (IAT) is performed to detect IgG antibodies.

Illustration of indirect antiglobulin testing (IAT).



Limitations

There are limitations even if the most sensitive screening methods are employed. If the antibody titer has dropped below the sensitivity level of the screening method being used, the antibody screen will be interpreted as negative. The screen also will not detect antibodies to low-incidence antigens that are not present on the screening cells.

Factors Affecting Sensitivity

- **Cell-to-serum ratio** – Antibody present within the test system in excess can cause false-negative results due to prozone, while antigen present in excess can cause false-negative results due to postzone. Increasing the amount of serum in the test system can help increase sensitivity when working with weak antibodies, but potentiators cannot be used.
- **pH** – Most antibodies react best at a neutral pH of 6.8-7.2. Acidifying the test system may enhance the detection of some examples of anti-M.
- **Temperature** – Pretransfusion compatibility testing focuses on detection of clinically significant antibodies, which generally react at 37 C or at AHG phase. IS and room temperature (RT) phases are often omitted to limit detection of insignificant cold antibodies.
- **Length of incubation** – If antigen and antibody are allowed too little contact time in the test system, enough cells may not become sensitized, leading to a negatively reacting screen. If the test system is allowed to incubate too long, bound antibody may dissociate. The time of incubation depends on the media used. Make sure the manufacturer's instructions are followed for all testing.

Basic Antibody Identification

Reactions seen at any phase of an antibody screen indicate the presence of an unexpected antibody. If the antibody screen is positive at any testing phase, careful consideration of the reactions seen can provide initial clues that begin the antibody identification process. Some initial questions to be asked are:

- What is the phase of reactivity where the reactions are occurring?
- Are the reactions true agglutination or rouleaux?
- Does patient have previously identified antibodies?

Patient History

Before starting antibody identification procedures, it is helpful to obtain a complete transfusion and/or pregnancy history on the patient for additional clues to the nature of the antibody problem. Recent transfusions may indicate recent antibody stimulation, and this also affects how to proceed with antigen typing. The patient's pregnancy history can reveal clues about recent antibody stimulation. Current and recent drug therapy information can explain the presence of antibodies that may have been passively transferred through the administration of IVIG or RhIG.

When preparing a panel for testing, an autocontrol should be tested with the panel cells. An autocontrol tests the patient's red cells against the patient's serum in the same manner as the antibody panel. This test is not normally performed with the antibody screen, but some laboratories still do. Most workers test an autocontrol with an antibody panel to help determine whether alloantibody or autoantibody specificity exists.

When reading the panel test results, it is important to grade all reactions consistently. Workers may choose to record their panel reactions on an antibody panel profile sheet. For all reagent antibody panels, an antigen profile sheet is provided that is specific for that particular panel lot. It is important to make sure the correct one is being used.

Panel Interpretation

Once results are recorded for each phase and negative reactions are confirmed with CCC, the panel can be interpreted.

Several steps aid in the interpretation of antibody identification testing. Each step will be covered.

- Phase of reactivity
- Reaction strength
- Autocontrol
- "Ruling out"
- Matching the pattern
- Rule of three
- Phenotype the patient

Phase of Reactivity

The phase where antibody reactivity is detected gives clues as to the immunoglobulin class of the antibody. IgM antibodies typically react at immediate spin or room temperature. IgG antibodies may be detected at 37 C, but preferentially react at IAT. Reactions at different phases may indicate more than one antibody and a combination of IgG and IgM antibodies.

Phase	Room Temperature/ Immediate Spin	37 C	IAT
Antibodies	Cold auto (I, H, IH) M, N P_1 Le^a, Le^b Lu^a	Potent cold auto D, E K	Rh Kell Duffy Kidd S, s Lu^b Xg^a
Immunoglobulin Class	IgM	IgG	IgG
Clinically Significant	No	Yes	Yes

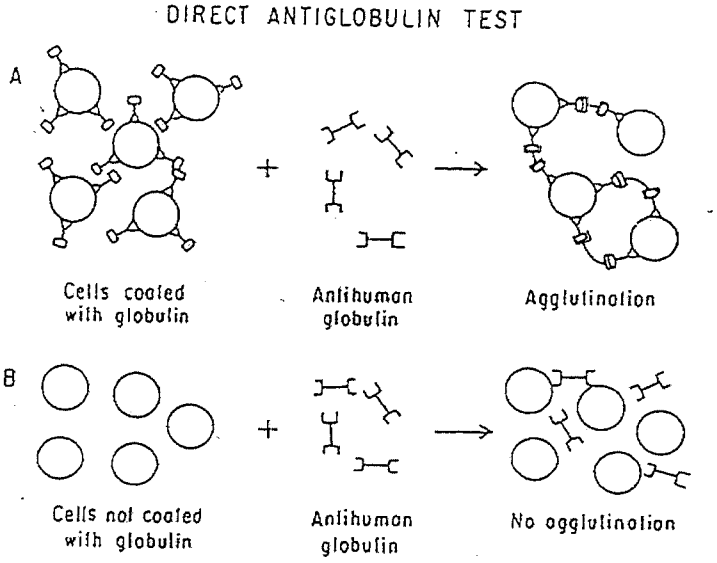
Adapted from Harmening D: Modern *blood banking and transfusion practices,* ed 5, Philadelphia, 2005, FA Davis.

Reaction Strength

Reaction strength is a clue to the number of antibodies present. Reactions of varying strengths suggest more than one antibody; however, varying strengths could also be due to dosage. One important thing to note regarding reaction strength is the strength of the reaction does not indicate the significance of the antibody, only the amount of antibody available to participate in the reaction.

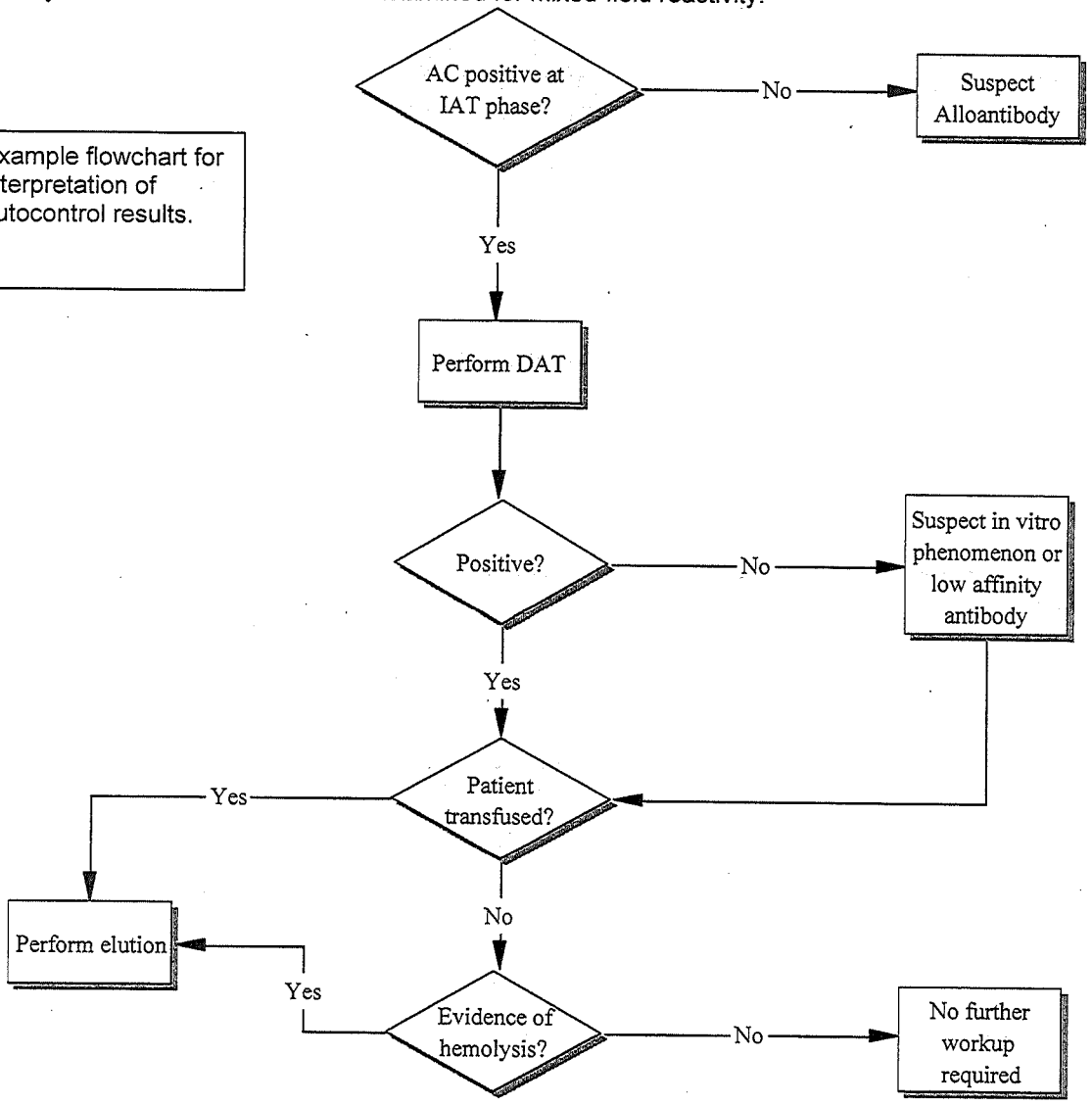
Autocontrol and DAT

The autocontrol helps determine the nature of the antibody in the patient's serum. If the autocontrol is negative, an alloantibody would be suspected. A positive autocontrol would indicate the presence of an autoantibody. When the autocontrol tests positive, a direct antiglobulin test (DAT) should be performed to determine the type of globulin coating the patient's cells. If the patient has recently been transfused, a delayed transfusion reaction should be suspected, especially if a mixed field reaction is noted when testing the DAT.



It is important to be able to distinguish between the autocontrol and the DAT. There are occasions where the autocontrol is positive while the DAT is negative. An autocontrol tests the patient's serum and cells along with enhancement media and a DAT is a one-step test to detect globulins bound *in vivo*. The enhancement media used in the autocontrol may cause reactivity that is only an *in vitro* phenomenon. If the autocontrol is positive and the DAT is negative, the panel should be repeated using a different type of potentiator or no enhancement media using a 30-minute incubation time. However, if the patient has been recently transfused, the autocontrol could be positive and the DAT negative due to a low affinity antibody. The autocontrol should be examined for mixed-field reactivity.

Example flowchart for interpretation of autocontrol results.



Ruling Out

Panel cells that give negative reactions in all phases can be used to exclude, or "rule out", antibodies. The process begins with examining the first nonreactive cell on the panel. Looking across the panel, place a line through the antigen specificity that is positive (+) on the panel. If an antigen is present on the cell and the serum did not react with the cell, the presence of the corresponding antibody may be excluded. Only cells (preferably in-date) with presumed double-dose antigen expression should be used in the case where the antibody has weak reactivity or is showing dosage. This process is continued with each nonreactive cell to exclude additional specificities. Ruling out with expired panel cells carries certain obvious risks and would need to be validated.

It is important to remember that ruling out is only a provisional step, especially if cells with single-dose antigen expression were used. The practice of excluding antibodies using only cells with presumed double-dose antigen expression is recommended whenever possible by most textbooks, but institutional policy must be followed.

It is acceptable to use single-dose cells for antigens that are not very prevalent in the population, such as K, or when there are multiple antibodies in a sample making it difficult to rule out or exclude with cells having presumed double-dose antigen expression.

Matching the Reaction Pattern

After the exclusion process is completed, the next step is to look at the reactions that are positive and match the pattern. If a single antibody is present, the pattern will match one of the antigen columns. If specificities remain that have not been excluded, additional testing is required.

Probability of Reactivity

To ensure the pattern of reactivity seen in the antibody identification testing is not due to chance alone, conclusive evidence must be derived by testing sufficient antigen-positive and antigen-negative cells. Many labs require a probability (p) value of 0.05 or less. A (p) value of 0.05 means there is a 5% chance the observed pattern happened by chance alone, or the correct antibody is identified 95% of the time.

A standard approach has been to require for each antibody specificity identified, 3 antigen-positive cells that react, and 3 antigen-negative cells that fail to react. A more liberal approach uses 2 antigen-positive and 3 antigen-negative cells The AABB *Immunohematology Reference Laboratory Standards* require 2 antigen-positive and 2 antigen-negative cells. No matter what approach is chosen, workers must follow their institution's policy.

Phenotype of the Patient

To confirm antibody identification, the patient's red cells should be phenotyped to ensure they are negative for the antigen corresponding to the antibody identified. Individuals should not make alloantibodies to antigens they possess. However, there are exceptions such as partial D and e variant.

Testing should be performed only if the patient has not been recently transfused. Patients with a positive DAT present a further complication if the antigen to be tested requires the IAT technique. One way to bypass this is to use monoclonal IgM reagents that do not require an IAT phase. Monoclonal typing reagents are available for some blood group system antigens such as Rh, K, and Kidd.

Complex/Autoantibodies

Multiple Antibodies

Patient serum containing two or more alloantibodies may make interpretation of test results difficult. Some clues that indicate multiple antibody reactivity include:

- The observed pattern does not fit that of a single antibody
- Reactivity is present at different test phases
- Reactions have varying strength of reactivity
- No obvious pattern is seen after performing exclusions
- Unexpected reactions occur when trying to confirm the specificity of a suspected single antibody

The following are tools that are helpful in clearing up the serologic picture:

- Determine if the pattern fits any combined specificities
- Evaluate each phase separately
- Test selected cells to eliminate some specificities
- Test cells with strong antigen expression or increase the sensitivity of the test system
- Use enzymes and/or chemicals
- Phenotype the patient

Selected Cell Panels

Selected cells are chosen from other panels to help rule in or rule out antibodies. When using selected cells from the same panel manufacturer, one important precaution is to ensure any selected cells are not the same donor source as any cells on the original panel. Most manufacturers give panel cells a donor number or code. One last note is to remember to use the screening cell results – the screen may have the selected cell you need!

Enzymes and Chemicals

Enzymes both eliminate and enhance antibody activity. Enzymes work by removing sialic acid residues from the RBC membrane, thereby removing some antigens and exposing others. Enzyme-treated cells should not tested with a potentiator and are read only at AHG phase to avoid false-positive reactions. DTT, trypsin, and alpha chymotrypsin are some chemicals that are usually used by reference laboratories in addition to enzymes.

Effect of Enzymes on Selected Blood Group Systems Antigens

Enhanced	Inactivated
ABO	MNS
Rh	Duffy
Kell	Xg^a
Kidd	
Lewis	
P_1	
I	

Antibodies to High Frequency Antigens

An antibody to a high-incidence antigen should be suspected when **all** reagent red cells are reactive, but the autocontrol is negative. The strength and the reactive test phase are usually uniform for all cells tested, although some antibodies such as Knops system antibodies can give varying reactions. Resolution can be difficult in the case of this type of antibody. Some helpful tactics are:

The patient's nationality or race	Anti-U and anti-Js^b should be considered if the patient is a Black person
System null cells	DTT-treatment makes K_o cells that aides in identification of anti-k, -Kp^b, -Js^b, -Ku (rare antibody
Enzyme-treated cells	Helps in identification of anti-Ch, -Rg, -JMH, -Yt^a, -Ge2, In^b, etc.

A sample with this type of reactivity is usually referred to a reference laboratory for further workup and identification of antibody specificity.

Anti-"HTLA"

HTLA or "high-titer, low-avidity" antibodies are usually directed to a high-incidence antigen, but they have characteristic weak reactivity that is sometimes displayed at a high titer, despite weak reaction strength. These antibodies react at IAT phase with inconsistent, "nebulous" reactions. While HTLA antibodies are usually not clinically significant, they can mask the presence of clinically significant alloantibodies. Recently though, these antigens and antibodies have taken on some importance in certain diseases such as SLE, AIHA, and CAD.

Neutralization Technique

Neutralization may be helpful in identification procedures with some antibody specificities, including HTLAs. Neutralization utilizes a substance that has antigenic substances similar to RBC antigens. These substances can be used to neutralize antibodies in serum, allowing for separation of antibodies.

Antibody	Substance Source
Anti-P_1	Pigeon egg whites, hydatid cyst fluid, turtledoves' egg whites
Anti-Lewis	Human saliva (from secretors), human plasma or serum
Anti-Ch, -Rg	Human plasma or serum
Anti-Sd^a	Human* and guinea pig urine**
Anti-I	Human breast milk

*Human urine is from Sd(a+) non-pregnant females since some women lose or have weakened Sd^a antigen and substance during pregnancy
**Highest Sd^a substance is in guinea pig urine

Antibodies to Low-incidence Antigens

Antibodies to low-incidence antigens are usually found in sera of multi-transfused or multiparous patients and serum of patients with warm reactive autoantibodies. Occasionally, they may occur as the only specificity, and should be suspected when the antibody screen is negative and a crossmatch is positive, or a baby has unexpected jaundice or a positive DAT, in the case of HDN, and the mother's antibody detection test is negative. Also, one reactive screening cell and nonreactive panel or a panel with one reactive cell suggests this type of antibody. A panel may be tested if the screen is negative but the patient has a history of an antibody. There is no need to identify an antibody directed to a low-incidence antigen, in the case of transfusion, but all other alloantibody specificities should still be ruled out. Blood that is crossmatch compatible through IAT phase is acceptable for transfusion. However, for HDN, it might be useful to identify the antibody for future pregnancies.

Autoantibodies

The first clue an autoantibody is present in the serum is reactions with most or all cells tested, and a positive DAT or autocontrol. The patient diagnosis and medication history are helpful to determine what type of autoantibody may be present. Since the primary goal of investigating the serum/plasma of these patients is detection and identification of clinically significant alloantibodies, techniques that detect and identify any underlying clinically significant alloantibodies must be used.

Cold Autoantibodies

Typical reactions are seen at IS phase, diminish through 37 C, with negative or weak reactions at IAT. Some cold autoantibodies can persist through the AHG phase due to binding at the IS phase that is not dispersed. Some pathologic cold autoantibodies can have high thermal amplitude, reacting at 30 C or above. The autocontrol is usually positive with polyspecific antihuman globulin reagents and negative with anti-IgG. The DAT is positive only with anti-complement.

Differentiating between autoantibody vs. alloantibody is helpful in determining the additional techniques that should be used. A quick way is to perform a "mini-cold" panel. A "mini-cold" panel utilizes screening cells, back typing cells, and group O cord cells (which have a weaker expression of H antigen than group O adult cells) to determine the specificity of a cold antibody.

Example #1
Group O patient with cold autoanti-I. Cord cells are negative. Testing with group A cells was not performed because of existing anti-A.

	SC I	SC II	AC	Cord	Cord	A_1	A_2
4 C	3+	3+	3+	0	0	NT	NT

Example #2
Group A_1 patient with cold autoanti-IH.

	SC I	SC II	AC	Cord	Cord	A_1	A_2
4 C	3+	3+	0-w+	1-2+	1-2+	0-w+	2+

Example #3
Group B patient with probable cold alloantibody. Cord cells possess antigens other than i. The first cord sample is reactive most likely due to the same antibody causing reactions with screening cell #1. Examples are anti-M, -P_1, -Le^a, -Le^b.

	SC I	SC II	AC	Cord	Cord	A_1	A_2
4 C	3+	0	0	3+	0	NT	NT

When a cold autoantibody is causing interference in a test system, the primary goal should be to circumvent the cold autoantibody to allow detection of clinically significant alloantibodies. Some helpful techniques are:

- Skip reading at IS and RT phases
- Perform cold autoadsorption (when patient has not been recently transfused)
- Use prewarmed technique (when autoadsorption cannot be performed)
- Use anti-IgG instead of polyspecific AHG
- Perform RESt adsorption (use with caution since it can adsorb out or weaken anti-B and other alloantibodies – see package insert)

Warm Autoantibodies

A warm autoantibody should be suspected when serum reacts with virtually all cells tested, the autocontrol is positive, and the DAT is positive. When dealing with a warm autoantibody, the goal is to determine what immunoglobulin (IgG, IgM, or both) is coating the patient's cells and detect and identify underlying clinically significant alloantibodies since they can be masked by the reactive warm autoantibody. Warm autoantibody activity tends to be enhanced with potentiators. Warm autoantibodies frequently react as if they are directed to antigens in the Rh system but if they show specificity, it is most often anti-e.

When the DAT is performed, it is usually positive due to IgG, but complement may also be present. There are rare examples of warm autoantibodies due to complement only. Most do not have a specificity, but a few with Gerbich specificity have been reported. It is important to differentiate a positive DAT from a positive DAT with mixed-field reactivity. When mixed-field reactivity is present, the implications take on a different meaning in a recently transfused patient. The patient could be forming new alloantibody, which appears as panagglutinin activity, especially if the new antibody is directed to a high-incidence antigen. In either case, an elution should be performed, especially in recently transfused patients to determine what antibody is coating the patient's red cells.

Elution

An elution is a technique to dissociate IgG antibodies from sensitized red cells. The antibodies attached to red cells are released, concentrated, and purified. The recovered antibody is called an eluate. It is important to test the "last wash" before preparing an eluate, or in parallel with the eluate, to check for unbound residual protein. The last wash must be nonreactive for a valid interpretation of eluate results. There are various methods used to perform an elution including:

- Temperature variation
- pH manipulation
- Chemical manipulation

When the eluate from a patient with a warm autoantibody is tested, it is usually reactive with all cells tested. Nonreactive eluates can also occur if the patient has made an antibody to a medication or if nonspecific binding of proteins to RBC membranes has occurred. In both of these instances, the patient can present serologically similar to a patient with a warm autoantibody. In a recently transfused patient, the eluate may show alloantibody specificity and/or warm autoantibody.

Adsorption

Adsorption is a technique used to remove selected antibodies from the serum by adding target antigen and allowing the antibody to bind to the antigen. The patient's serum is incubated with appropriate red cells under optimal conditions. For warm autoantibody removal, incubation is performed at 37 C, and for cold adsorption, the cells and serum are incubated at 4 C.

There are different types of adsorptions, the limiting factors being the transfusion history of the patient and the amount of sample available.

An autoadsorption uses the patient's own cells as the source of the target antigen. This is usually the optimal adsorption to perform because there is no chance of removing alloantibodies. Patients transfused in the last 3 months do not qualify for autoadsorption because of the potential for transfused red cells to be present in the patient's circulation. Severely anemic patients may not have enough red cells to carry out the procedure.

If an autoadsorption cannot be performed, allogeneic adsorption is the next option. Generally, the Rh and Kidd systems are tested and matched when choosing allogeneic cells. Adsorbing cells can be pretreated with enzyme to remove some antigens. Enzyme pretreatment enhances antibody uptake and also helps make the appropriate phenotype of adsorbing cells easier to find. There are **rare** examples of autoantibodies that do not react with enzyme treated red cells; an example is antoanti-En^a FS (FS for ficin sensitive).

If the patient's phenotype can be determined, cells that are phenotypically similar are used in alloadsorption. If the patient's phenotype cannot be determined, differential or "triple" adsorptions is performed. In this procedure, three separate adsorptions are performed using R_1R_1, R_2R_2, and rr cells. Among the three adsorbing cells, one cell must be negative for K, another negative for Jk^a, and the third negative for Jk^b. Each aliquot of cells is treated with enzyme to destroy certain antigens (MNSs and Duffy) and aid in antibody uptake.