

IV. Principles of Serological Testing in Immunohematology

A. Blood Group Antigens and Antibodies

1. Blood group antigens are structures embedded in or protruding from RBCs, WBCs and Platelets.
2. Exposure to blood group antigens is usually the result of a prior transfusion or pregnancy causing exposure of the individual to antigens they do not possess.
3. An individual may form antibodies to the antigens on these types of cells which they lack.
 - a. Immunohematology is concerned with the detection of IgG and IgM class antibodies.
 - b. IgM antibodies are usually naturally occurring, RT agglutinins, easily detected by routine procedures.
 - c. IgG are immune antibodies produced as a result of exposure to the antigen and require a special serological test (the Coomb's procedure) to detect their presence.
4. Immunohematology uses some basic serological tests to detect the presence of antibodies in an individual's serum and/or antigens on an individual's cells.
5. The detection of antigens is routinely used to determine a person's blood group and D type.
6. Antibody detection tests are used to confirm the ABO antigen typing and to detect "unexpected antibodies" or immune antibodies in the serum which may cause the destruction or hemolysis of transfused cells containing the antigen.

B. Dynamics of Antigen-Antibody Reactions

1. Union of antigen with its specific antibody depends upon structure and charge of the molecule.
 - a. Lock and key.
 - b. Opposite charges on antigen and antibody create attraction forces.
 - c. Reaction is a reversible bimolecular reaction.
2. Physical forces hold antigen and antibody together.
 - a. Physical forces are weak.
 - b. The attractive forces vary in strength with changes in pH, ionic strength, temperature, and nature of the solvent.
 - c. Blood group antigens and antibodies bind to each other until a dynamic equilibrium is reached in which as many bonds are formed as are disrupted.
 - d. Represented by a bell shaped curve based on concentration of the reactants:
 - 1) Prozone (antibody excess)
 - 2) Zone of equivalence, optimal proportion of antigen and antibody.
 - 3) Postzone (antigen excess).

C. Detection of Antigen-Antibody Reactions in Immunohematology

1. **Agglutination** is the most common procedure used in immunohematology.
 - a. RBCs have antigenic determinants which combine with antibodies present in the test serum added.
 - b. Antibody bridges form with antigenic determinants on adjacent cells resulting in agglutination
2. **Hemolysis** represents destruction of RBC membrane and when detected, hemolysis is considered a positive result.
3. Solid Phase Adherence is used to detect and identify antigens or antibodies.
 - a. Methodology primarily used in donor screening.
 - b. Uses microplates coated with RBC or antibody to which serum or RBCs are added.
 - c. If antigen-antibody reaction occurs, the cells adhere to the sides of the well, if no reaction occurs the cells settle to the bottom of the well.

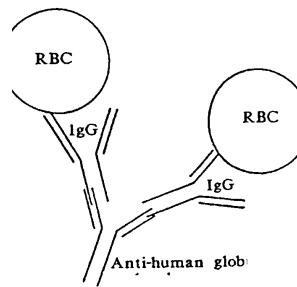
D. Principle and Variables Involved In Agglutination Reactions

1. Two stages involved in agglutination reaction.
 - a. The **first stage** of agglutination is **sensitization**, attachment of antibody on to the corresponding antigen site on the RBC membrane.
 - b. The **second stage** is the formation of bridges between the sensitized cells.
 - 1) IgG antibodies have two antigen binding sites, one site will bind to antigen on an RBC and the other site will bind to antigen on a different RBC resulting in the formation of a lattice.
 - 2) **Lattice formation** results in hemagglutination.
2. Variables affecting first stage of agglutination.
 - a. **Antigen-antibody ratio** (serum to cell ratio) is very critical.
 - 1) Optimal amount of serum must be determined.
 - 2) Increasing amount of serum increases number of antibody molecules coating the cell.
 - b. **pH** - The reactivity of the majority of blood group antibodies is optimal at a **pH** of 6.5 - 7.5, for routine blood bank procedures, a pH of 7.0 should be used.
 - c. **Ionic strength of suspending medium**
 - 1) Saline partially neutralizes oppositely charged sites on antigen and antibody molecules which hinders the association of antigens and antibodies.
 - 2) Low ionic strength saline is a saline solution with a lower salt concentration which enhances uptake of antibody onto the cells.

- 3) Albumin unless used under low ionic conditions does little to affect antibody uptake on to the cell, rather it influences the second stage of hemagglutination.
 - d. **Temperature requirements** differ depending upon antibody class involved.
 - 1) Antibodies of the IgG class attach to antigens optimally at 37 C.
 - 2) IgM antibodies attach to antigens preferentially at RT or below (4 - 22 C).
 - e. **Incubation time**
 - 1) Antigen-antibody reactions have an optimal time of incubation that favors maximal binding of antibody to antigen.
 - 2) Enhancement agents increase antibody uptake onto the cell thereby decreasing the incubation time.
4. Variables affecting the second stage.
- a. **Immunoglobulin class**
 - 1) IgG is a small monomer with the ability to sensitize RBCs, but not agglutinate.
 - 2) IgM is a large pentamer with 10 antigen binding sites, very easily agglutinates RBCs.
 - b. **Antigen Sites** antigen sites may be sparsely distributed or present in low numbers which may result in no agglutination.
 - c. **Electrostatic Repulsion Forces**
 - 1) Lattice formation depends upon overcoming the electrostatic forces of RBCs.
 - 2) The *zeta potential* is the term used to describe the electrical forces which keep RBCs away from each other serologic methods are aimed at reducing the zeta potential.

E. Principle of the Antiglobulin Test

1. Hemagglutination is the most common method used in blood banking for the detection and identification of blood group antigens and antibodies but in some cases the short IgG antibody molecule can sensitize RBCs but cannot produce agglutination.
 - a. The small size of the IgG antibody molecules makes them unable to overcome the forces that cause RBCs to repel each other.
 - b. If undetected, IgG antibodies can cause coating and destruction of transfused RBCs.
2. In 1945 Coombs, Mourant and Race described a test for detecting nonagglutinating, coating antibodies now called the *anti-human globulin (AHG or Coomb's)* test.
 - a. A reagent antibody to IgG (anti-IgG) and/or complement is produced (AHG serum) and is added to test cell after incubation of the cell with serum or antisera.
 - b. If the cells have been sensitized (coated) with IgG antibodies during the incubation period the AHG will attached the second antigen binding site of the IgG molecules coating the cells, causing agglutination.



RBC coated with IgG antibodies, anti-human globulin serum (anti-IgG) binds to the IgG antibodies coating the RBC resulting in lattice formation, ie, agglutination.

****Memorize the principle and be able to draw this diagram for future exams****

3. Importance

- a. Prior to 1945 only complete, IgM antibodies could be detected and only a few blood group antigens had been identified.
- b. ABO antibodies and antigens were detected but hemolysis of transfused cells still occurred even when the blood was ABO compatible.
- c. This test provided the explanation, in-vitro testing could detect agglutination caused by IgM antibodies but could not detect sensitization by the IgG antibodies.
- d. It was determined that certain IgG antibodies are capable of causing destruction of transfused RBCs and also capable of crossing the placenta and destroying fetal RBCs.
- e. It is now a requirement that antibody detection test include an antiglobulin phase of testing.

F. Two Methods of Preparation of the Antiglobulin Serum

1. Animal Inoculation

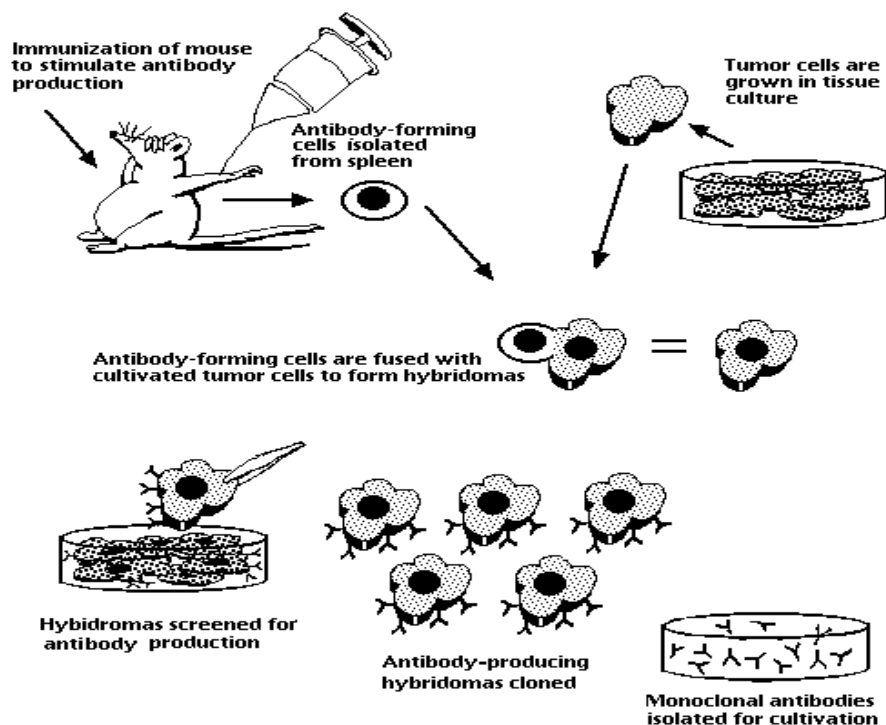
- a. Human serum (or purified globulin from human serum) is injected into a suitable laboratory animal, rabbits most commonly used, the animal responds to the injection of foreign antigen by producing antibodies directed against human globulin (anti-human globulin or AHG).
- b. Animal is bled and unwanted antibodies are adsorbed out (removed).
- c. AHG will react with human globulin molecules (antibody) either bound to red blood cells or free in serum.

2. Monoclonal Reagent Preparation

- a. Animal (mouse most popular) is injected with highly purified immunogens such as IgG, IgA, IgM, C3 or C4, causing the animal to produce antibodies against the immunogen injected.

- b. A **hybridoma** is prepared by the artificial fusion of the animal immunoglobulin secreting cell (mouse spleen cell) and a myeloma cell, which produces massive quantities of antibody.
 - 1) The myeloma cell produces antibody without specificity.
 - 2) Fusion of the myeloma cell with the antibody producing spleen cell results in an immortal cell which produces massive quantities of antibody with **one** specificity.
- c. The antibody containing fluid is harvested from immunosecreting cell lines (hybridoma).
- d. The hybridoma technology is rapidly changing the types of reagents used in blood banking, reducing and, eventually, eliminating the need for reagents produced from animals and humans.

Monoclonal Antibody Production



G. Types of Anti-Human Globulin (AHG) Reagents

1. Polyspecific AHG

- a. *Polyspecific AHG consist of a pool of anti-human IgG and anti-C3b and C3d.*
- b. May be produced by hybridomas, rabbits, or a mixture.
- c. Used for routine compatibility testing, antibody identification and DAT.
- d. Most important function is to detect IgG antibodies coating the cells.
- e. The importance of the presence of anti-complement in AHG serum is very controversial for routine compatibility testing.
- f. Antibodies which are detectable only by their ability to bind complement are rare, so it's not as useful for compatibility testing as many false positive reactions occur.

2. **Anti-IgG**

- a. *Major component is antibody to human gamma chains (anti-IgG) contains no anti-complement.*
- b. Used as alternative to polyspecific AHG in routine blood bank procedures.
- c. Advantage of utilizing anti-IgG is that it eliminates the false positives frequently obtained when using polyspecific AHG due to complement and non-specific cold reacting antibodies yet will detect clinically significant alloantibodies.

3. **Anti-C3b, Anti-C3d**

- a. *Contains anti-complement antibodies to detect complement coating the cells.*
- b. Used for classifying the coating proteins on RBCs to determine if IgG, complement, or both are present.
- c. Primary importance is for classification of autoimmune hemolytic anemias and drug induced hemolysis of RBCs.

H. Antiglobulin Techniques

1. ***Direct Antiglobulin Test (DAT)*** is used to demonstrate or detect *in-vivo* coating of RBCs with globulin, particularly IgG and/or C3.
 - a. One drop of a patient's cell suspension is washed 3 times to remove all contaminating proteins.
 - b. AHG is added **directly** to the washed cells, centrifuged and observed for agglutination, which is indicative of IgG antibodies coating the cells, the AHG serum will bind to the IgG on the cells forming lattices = agglutination.
2. ***Indirect Antiglobulin Test (IAT)*** detects *in-vitro* sensitization with antibody complement or both.
 - a. Patient serum (or special types of anti-serums) are added to cells and incubated at 37 C (body temperature) for a specified time.
 - b. Read after incubation because some IgG antibodies may coat the RBCs heavily enough after incubation to cause agglutination.
 - c. The RBCs are washed free of contaminating protein and AHG serum is added, if the cells have been coated with IgG during the incubation phase the anti-antibody in the AHG will form lattices which resulting in agglutination.
3. When a patient has an antibody identified in their serum it is critical to find donor units which lack the antigen, typing the donor may require the IAT.
4. The crossmatch procedure also requires the IAT, patient serum is incubated with donor cells, washed and tested with AHG serum. Agglutination indicates that the unit cannot be given to the patient (it is incompatible).

I. **False negative results in the DAT and IAT. MEMORIZE!**

1. Failure to wash red blood cells adequately.
2. Testing interrupted or delayed during the washing procedure.
3. Loss of activity in AHG reagents
4. Failure to add AHG.
5. Improper centrifugation
 - a. Under centrifugation
 - b. Over centrifugation
6. Incorrect concentration of red blood cells in test system.
7. Prozone reactions
8. Negative DAT may not necessarily mean absence of coating globulins.
 - a. Poly and anti-IgG will detect approximately 200 molecules of IgG per cell.
 - b. Presence of weak antibody may go undetected.
9. If a negative reaction is obtained, IgG coated reagent RBCs (check cells), are added to the test system, agglutination ensures that the AHG serum is active.
10. In the DAT complement coating may not be apparent as agglutination upon immediate reading.
11. False negatives in the IAT (IAT and DAT are different).
 - a. Red cells and serum lose reactivity if improperly stored.
 - b. Occasional examples of anti-Jk^a and anti-Jk^b may be detected only in the presence of active complement.
 - c. Temperature and incubation time affect attachment of antibody or complement to the red blood cells.
 - d. Optimal proportion of serum to cells should be achieved.

J. **False positive results in the DAT and IAT. MEMORIZE!**

1. Red cells may be agglutinated before they are washed.
2. Saline stored in glass bottles may contain colloidal silica particles that can leach from the container into the saline.
3. Improperly cleansed glassware.
4. Overcentrifugation
5. Improperly prepared AHG reagents.
6. DAT - additional considerations
 - a. Complement components, primarily C4, may nonspecifically bind to red blood cells from clotted blood samples kept at 4 C.
 - b. False positive DAT results may occur with blood collected into tubes containing silicone gel.
 - c. Blood samples collected from intravenous fluid lines used to administer solutions.
 - d. Septicemia in a patient or bacterial contamination of stored specimens.

7. IAT - additional considerations

- a. Red cells coated with IgG cannot be tested with antisera that react only by IAT, such as D^u or anti-Fy^a.
- b. Procedures are available for removing IgG from in vivo coated red blood cells.

K. Role of complement in the antiglobulin reactions.

1. Complement-binding antibodies attach complement to the cell surface when they react with red cell antigens.
 - a. Some blood group antibodies bind complement to the red cell membrane.
 - b. Clinical significance of complement binding antibodies is variable.
2. Immune complexes present in plasma activate complement components that may adsorb to red blood cells in a non-specific manner.
3. Complement coated red cells may or may not proceed to hemolysis.