Antigen / Antibody Interactions in Immunohematology

I. Definitions

A. **Immunohematology**: A branch of immunology pertaining to the antigens of blood cells and their corresponding antibodies.

B. **Antigen**: A substance that is recognized by the body as foreign and can elicit an immune response. In Immunohematology, the key antigens of interest are found on the red blood cell (RBC) membrane. Abbreviated “Ag”.

C. **Antibody** (a.k.a. immunoglobulin): A protein secreted by plasma cells in response to, and interacting with a specific antigen. The purpose of the antibody is to promote the removal of the antigen. Abbreviated “Ab”.

   1. In Immunohematology, the primary focus is on “clinically significant” antibodies that reduce the lifespan of the RBC.

   2. These are primarily IgG antibodies; some IgM.

II. Mechanisms of Antigen – Antibody Interaction

A. The Fab portion of the antibody is complementary to the structure of the antigen, allowing the antigen and antibody to bind together in “lock and key” fit.

B. Antibody promotes removal of the antigen by:

   1. Coating antigen to facilitate phagocytosis by macrophages. Macrophages have cell surface receptors for the Fc portion of the immunoglobulin molecule. Macrophage will bind to immunoglobulin that is bound to the antigen, and then engulfs the antigen.

   2. Activating **complement**.

      a. Phagocytes have receptors for the C3b component of complement. If an antibody has activated complement, and C3b is on the antigen surface, the phagocytes will bind to the complement and engulf the antigen.

      b. Cell lysis. If the complement cascade goes to completion, a pore will be created in the cell membrane causing the cell to burst. An antibody that causes RBCs to lyse is called a **hemolysin**.
III. *In Vitro* Detection of Antigens and Antibodies

A. Antigens are detected by using reagents that contain the corresponding antibody. Antibodies are detected by using reagents (usually RBCs) that possess the target antigen.

B. Ag/Ab reactions in immunohematology tests are visualized by:

1. **Agglutination**: An immunoglobulin molecule binds to antigens on opposing RBCs, creating a lattice between the cells. The RBCs appear to clump together. An antibody that causes agglutination is called an *agglutinin*.

   ![Agglutination](image)

2. **Hemolysis**: The antibody activates complement, leading to the lysis of the RBC. Hemolysis will only be seen if a serum specimen is used; anticoagulated specimens do not contain the calcium required for complement activation.

C. Factors that influence antigen-antibody reactions

1. **Antigen to antibody ratio** (i.e. prozone and postzone).

   a. Traditionally, testing is performed using a ratio of 2 drops of plasma (or serum) or anti-serum reagent containing antibodies to 1 drop of a 2-5% suspension of RBCs (antigen) in saline.

   b. The use of monoclonal antibody reagents and different methodologies may alter this ratio.

2. **Temperature**

   a. IgM - optimal reactivity at room temperature (20-25°C) and below (cold)

   b. IgG - optimal reactivity at 36-38°C (warm)
3. Reaction time

a. IgM antibodies tend to react immediately (no incubation time). May only need centrifugation in order to bring antigens in close proximity to allow for cross-linkage by antibodies.

b. IgG antibodies usually require incubation at 37°C for a period to achieve sensitization (uptake of antibody onto the RBC) and lattice formation.

c. If the reaction time is too short, there will not be time for Ag/Ab interaction and lattice formation (false negative).

d. Prolonging the reaction time favors the free antigen and antibody state, also resulting in false negative test results.

4. Ionic strength: In a normal saline solution, the Na⁺ and Cl⁻ ions cluster around and partially neutralize opposite charges on antigen and antibody molecules, which hinders the association of antigen and antibody.

a. Shielding effect – Ions of opposite charge block the Fab piece, preventing Ag/Ab binding. Effect can be reduced by lowering the ionic strength of the reaction medium which in turn increases the rate at which antigen-antibody complexes are formed.

b. Zeta potential – the difference in electrical charge between the surface of a cell and the outer layer of the ionic cloud that surrounds the cell in an electrolyte solution. Reducing the zeta potential by altering the test medium allows cells to draw closer together, allowing for increased lattice formation.

c. IgG antibodies (incomplete antibodies) often require the use of potentiators (a.k.a. enhancement media) to reduce zeta potential and the shielding effect. IgM antibodies (complete antibodies) usually do not.

5. Number and position of the antigen sites

a. More antigens are available to participate in a reaction when both parents pass along the same the gene for antigen expression (homozygous) than when different genes are inherited from each parent (heterozygous).

b. Antibodies that react stronger with cells having homozygous antigen expression than with cells having heterozygous expression are said to show dosage.

c. The position of one antigen in relationship to another may disrupt the ability of the antigen to bind with its corresponding antibody.
6. pH: Optimal activity between 6.5 and 7.5

D. Direct agglutination test

1. RBCs and antibody (from plasma/serum or reagent) are mixed together in a test tube or other reaction surface, such as a microtiter well.

2. Centrifugation is used to bring RBCs closer together to facilitate lattice formation.

3. Usually performed at room temperature. Called the immediate spin phase (IS). Some (IgG) antibodies require incubation at 37ºC before agglutination can be detected. This is called the 37º phase (37).

E. The Antiglobulin Test

1. Most IgG antibodies will not react in the immediate spin phase because it is performed at room temperature. While sensitization of the RBCs with antibody will take place at 37ºC, agglutination usually will not, as the IgG molecule is too small to bridge between RBCs. In order to bridge between sensitized (coated) RBCs, an additional reagent is required – anti-human globulin reagent. This is the antiglobulin phase of testing (AHG).

2. Two antiglobulin test methods:
   b. Indirect – RBCs are sensitized in vitro by incubating antigen and antibody together at 37ºC. May use patient’s RBCs or plasma/serum. Often requires the use of enhancement reagents to facilitate the reaction.

3. Mechanism
   a. Anti-human globulin reagent (AHG) is used to determine the presence of immunoglobulins or complement on the surface of red blood cells. Before performing the AHG test, the RBCs are usually washed repeatedly with saline to remove any trace of unbound globulin.
   b. AHG reagent is an IgG antibody directed against either the Fc portion of the human IgG molecule or the C3 or C4 component of complement.
   c. The Fab portion of the antibody in AHG reagent is directed at the Fc portion of the human IgG molecules attached to each of two separate RBCs. (or the AHG antibody is directed at complement coating two separate RBCs).
d. AHG antibodies bind to the human antibodies or complement, bridging the gap between sensitized red blood cells to create visible agglutination.

4. Anti-human globulin reagent (a.k.a. Coombs serum)

a. Made by injecting rabbits with human serum (globulins) which produce antibody to the foreign protein (AHG). Hybridoma technology is also used to produce some reagent.

b. Three main types of AHG:

1) Broad spectrum (polyspecific or polyvalent)
   a) Contains anti-IgG and anti-C₃d.
   b) Detects IgG and complement on red blood cells
      -Some IgM antibodies activate complement which would be detected at the AHG phase by the anti-C₃d.

2) Monospecific: Contains only one component- should be heavy chain specific to guard against cross reactivity.
   a) Anti-IgG - AABB requires that AHG reagent must contain anti-IgG when being used for antibody detection.
   b) Anti-IgM – rarely used
   c) Anti-IgA – rarely used

3) Monospecific anti-complement
   a) One or more of the complement components are present
      i. Anti-C₃ and C₄
      ii. Anti-C₃b, C₃d, C₄b
      iii. Anti-C₄d, C₃d
   b) Very few clinically significant antibodies require anti-complement for detection. The exception is some examples of Kidd antibodies.

5. Control of the AHG test

a. When performing the antiglobulin test by tube method, all negative tests must have Coombs Control Cells (a.k.a. Check Cells) added to confirm the reactivity of the test.
b. Must have a positive reaction with Coombs Control Cells (CC) for the antiglobulin test to be valid. If negative with CC, the test must be repeated from the beginning.

c. Coombs Control Cells prove:

1) The AHG reagent was added to the tube (the reagent often has green dye added to make it more visible in the test tube).

2) The AHG reagent was active

3) The wash step was adequate to remove unbound antibodies. (Unbound antibodies that remain in the test tube would react with the AHG reagent, effectively neutralizing the reagent)

d. Coombs Control Cells are RBCs that have been coated with either an IgG antibody or complement. The IgG or C₃b that is on CC reacts with the antibody in the AHG reagent and causes agglutination.

F. Gel Method (a.k.a. Column Agglutination)

1. Rather than using a test tube to conduct reactions, the gel method uses microtubules that are packed with dextran acrylamide gel. A reaction chamber sits above the microtubule; cells and/or plasma to be tested are added to the reaction chamber. The chamber/microtubule arrangement is packaged in a plastic card about the size of a credit card. Typically 6 chamber/microtubules are on each card.

2. Prepackaged reagents may be located at the interface of the reaction chamber and microtubule.

3. Once the antigens and antibodies have been given time to react in the reaction chamber, the card is centrifuged, forcing the RBCs into the gel layer.

4. If an antigen/antibody reaction has occurred, the agglutinated RBCs become trapped in the gel, as the agglutinates are too large to pass between the gel particles. This is considered a positive test.

5. If no antigen/antibody reaction has occurred, the individual RBCs pass through the gel, and form a pellet at the bottom of the microtubule. This would be considered a negative test.

6. The gel method may be used for both direct agglutination and antiglobulin tests.

7. The gel method can be easily automated.
G. Solid Phase Adherence Method

1. Microtiter plates are used as the platform for solid phase adherence testing. Red blood cell antigens of known phenotype are immobilized on microtiter wells. The patient’s plasma/serum to be tested is added.

2. Following incubation, a wash step is used to remove any antibodies that have not bound to the red blood cell antigens.

3. Typically, anti-IgG-coated indicator cells are added to aid in the detection of bound antibodies.

4. If an antigen/antibody reaction has occurred, the anti-IgG-coated indicator RBCs will be “trapped” in the matrix, forming a diffuse pattern throughout the well. The test is considered to be positive.

5. If no reaction has occurred, the indicator RBCs will form a dense pellet at the bottom of the well.

6. The solid phase adherence method is also readily automated.

Left Column wells A and B – Positive

All other wells – Negative
IV. Common Blood Bank Reagents

A. Anti-sera: Reagent contains antibodies. Used to detect antigens on RBCs.

1. Traditional Polyclonal
   a. Produced by immunizing donors, and then collecting the sera containing antibodies.
   b. Advantage - Reagent contains antibodies directed against multiple epitopes, so there is a lesser chance of missing a partial antigen.
   c. Disadvantages –
      1) Great variation in antibody titer between lots. Patient may test positive with one lot and negative with another.
      2) Greater cross-reactivity yielding false positive results.
      3) Since source is human, there is the risk of infectious disease (i.e. hepatitis, HIV)
      4) Ethics of purposefully stimulating antibody production in a previously non-immunized individual.

2. Monoclonal
   a. Produced by fusing mouse lymphocytes that are dedicated to one specific epitope with myeloma cells, which secrete large amounts of antibody. These hybridomas are tested and cell lines cultured to provide a long-lasting, abundant source of very pure, very specific antibody (immortal clones).
   b. Advantages
      1) No lot-to-lot variation in reactivity. Very consistent reactions.
      2) High titers of antibody can be produced.
      3) High specificity- no cross-reactions or non-specific reactions.
      4) High sensitivity- reacts with small amounts of antigen.
      5) Reduced risk of transmitting infectious diseases.
c. Disadvantage –

1) Contains antibody directed against only one epitope. If cells are lacking that epitope the antibody in the reagent will not react with those cells, giving a false negative reaction for that antigen.

2) To overcome this disadvantage, most monoclonal reagents are a blend of several monoclonal antibodies or a blend of monoclonal and polyclonal antibodies.

B. Red Blood Cell Reagents: Reagent possesses antigens; used to detect antibodies.

1. Suspended in a diluent/preservation solution that maintains antigens and retards hemolysis.

2. Traditional concentration for red blood cell reagents is between 2-5%, but will vary according to method being employed.

3. Because RBCs possess antigens from multiple antigen systems, one may get a false positive reaction if the plasma being tested contains unexpected antibodies to antigens from a system not under consideration.
   -For example, when determining the ABO group of an individual, B cell reagent (possessing B antigen) is used to look for anti-B in the individual’s plasma. If the individual’s plasma contains anti-M (from the MNSs system) and the reagent B cells also happen to possess the M antigen, the B reagent cells will test positive due to the M/anti-M reaction, and not to a B/anti-B reaction.

C. Enhancement Reagents (a.k.a. potentiators): Used in the indirect antiglobulin test to promote agglutination and in some instances increase the rate of sensitization.

1. Albumin
   a. High molecular weight protein
   b. Reduces the zeta potential / increases the dielectric constant which allows the RBCs to come in closer proximity facilitating cross-linking by antibodies.
   c. Allows for a reduced incubation time at 37°C (15-30 minutes vs. 30-60 minutes for a saline test system).

2. Low Ionic Strength Solution (LISS)
   a. Made of NaCl, glycine and albumin
b. Creates a low ionic environment which lowers the zeta potential

c. Promotes antibody uptake by the red cells (sensitization) by reducing the shielding effect

d. Incubation time reduced to 10-15 minutes.

e. **Most commonly used enhancement solution**; used in tube, gel and solid phase methods.

3. PEG

a. Polyethylene glycol in a low ionic strength medium (LISS). Has all the advantages of LISS **plus**:

b. Removes water from the test system which concentrates the antibodies, making it more likely that antigen and antibody will encounter each other.

c. More likely to detect weak antibodies than albumin or LISS.

d. Incubation time – 15 minutes.

4. Enzymes

a. Proteolytic substances that remove sialic acid residues from RBC membrane, thereby reducing surface charge. Enzymes also split polypeptide chains, which further exposes some antigens, but destroys other antigens.

b. May enhance the hemolytic activity of some complement dependant antibodies.

c. Ficin, papain, bromelin and trypsin are the enzymes most commonly used in blood banking, however enzymes are generally considered a special technique used only after routine methods have failed to yield definitive results.