Basics of Immunohematology

(Ag, Ab, C+ & F. affecting Ag Ab Reactions)

Dr. Nabajyoti Choudhury
Addl. Director & Head
Fortis memorial Research Center,
Gurgaon, Delhi (NCR), India

Antigens (1)

- Antibodies belong to a group of proteins called immunoglobulins, that have a common structure of two pairs of chains arranged symmetrically along the long axis. All antibodies are capable of specific combination with antigen

Antigens (2)

- Antigens - substances recognized by the body as foreign, causing the body to produce an antibody to react specifically with it.
- Antigens are chemically complex, (M Wt:10,000 d) & protein or polysaccharide in nature.
- There are specific antigenic determinant sites, or epitopes, which are those portions of the antigen that reacts specifically with the antibody

Antigens (3)

- Hapten: Small molecule <1000 d which alone cannot elicit immune response
- Carrier: Hapten can elicit immune response if complexed with large molecule (called carrier)
- Immunogen: Antigen capable of eliciting an immune response without prior coupling to a second molecule are called imunogens.
- Immunogenicity or antigenicity: The degree to which a substance is likely to elicit an immune response in a given category of host

Antibodies (1)

- Polypeptide chains: Ab belongs to a group of protiens called immunoglobulins (Ig), having common structure of 2 pairs of chains.
- All Abs are capable of specific combination with Ags.
- Two identical L chains (220 amino A) & 2 identical H chains (440 amino A)
- Isotype: Distinctive amino A that characterize individual classes [alpha, delta, epsilon, gamma, mu]

Antibodies (2): Ig classes

- **IgG**: Gamma H chains; Main Ig in blood & extravascular fluid; Single basic Ig of 2-L & 2-H chains; Crosses placenta; Produces IgG in secondary response; Quantification in blood IgG1>IgG2>IgG3>IgG4

- **IgM:** Mu H chain; Pentamer; 10 combining sites; 1st Ab produced by fetal immune system; Only in blood; Effective Ab agglutinin and fixes complemt; 2-ME & DTT cleaves IgM

Antibodies (3): Ig classes

- IgA: Alpha H chain; Exist as single units or as polymers; Present in blood, epithelial secretions (saliva, tears, GI/ respiratory fluid); Non agglutinin & complmt binding; Two types: IgA1 & IgA2
- IgD: Delta H chain; Mainly on membrane of B lymph acts as receptor; No blood Gp Ab.
- IgE: Epsilon H chain; Very low conc. in serum; Bound to basophil/ mast cells; Release histamin; Causes edema, rashes, increased secretion, respiratory constriction

Antibodies (4): Types of Ab

- Allotypes: The Aminoacid sequence in certain segments in the contant regions of Ig chain varies to produce heritable trait. (24 in no.)
- Idiotypes: Antibody mol. of individual specificity have a unique AA sequence in variable domains that comprise Ag binding portion of the molecule. AA sequence gives 3 dimensional configuration that allows Ag to interact with specific Ag. (*antigenically unique feature of Ab is called idiotype*)

Complements (1)

- Complement is the term applied to a system of 25-30 serum & membrane proteins that act in a cascading manner- similar to the coagulation, fibrinolytic and kinin system- to produce numerous biological effects.
- It has 3 major roles:

  # promotion of acute inflammatory events,

  # alteration of surface for phagocytosis

  # modification of cell surface for lysis

Complements (2)

- Defensive action (destruction of bacteria, protects against virus, eliminates protein complex, enhance immune events)

- For the host: (initiate inflammatory and immune processes harmful to host, mediate destruction of host cells)

Cytokines

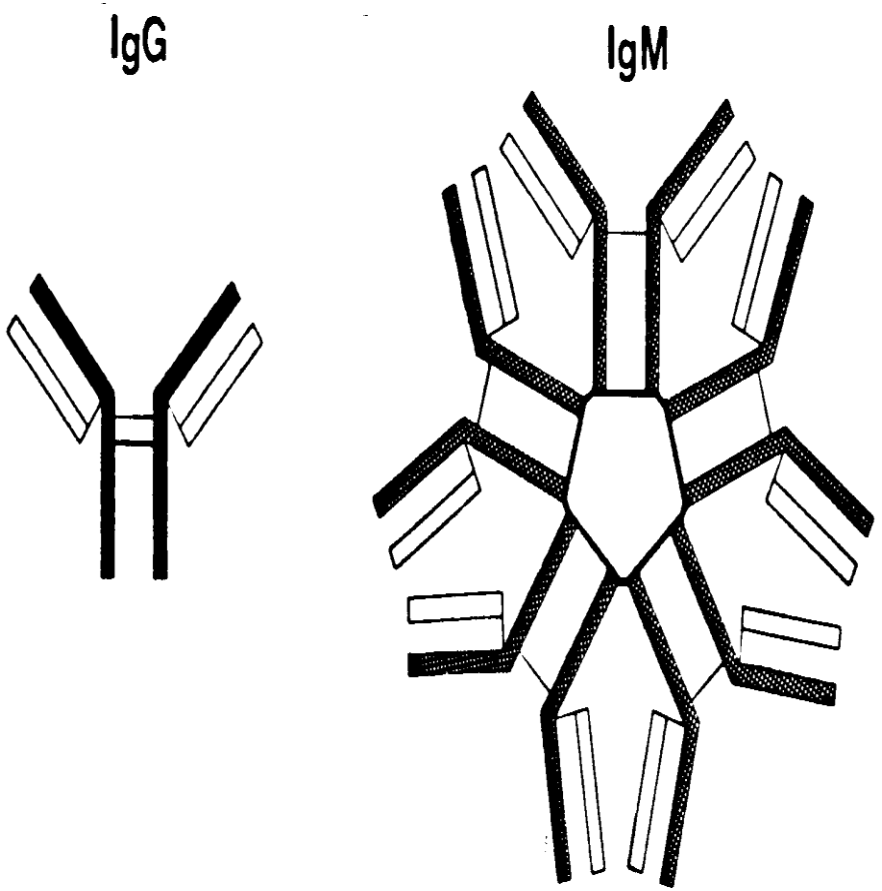
- Cytokines are a diverse group of intracellular signaling peptides and glycoprotein that have mol. wt. 6,000-60,000 d.
- Each cytokine is secreted by particular cell in response to different stimuli.
- Important functions: inflammation, tissue repair, cell activation, cell growth, fibrosis.
- Cytokines in TM: FNHTR, HTR, stem cell collection, immunomodulation.

Blood Group Antigens

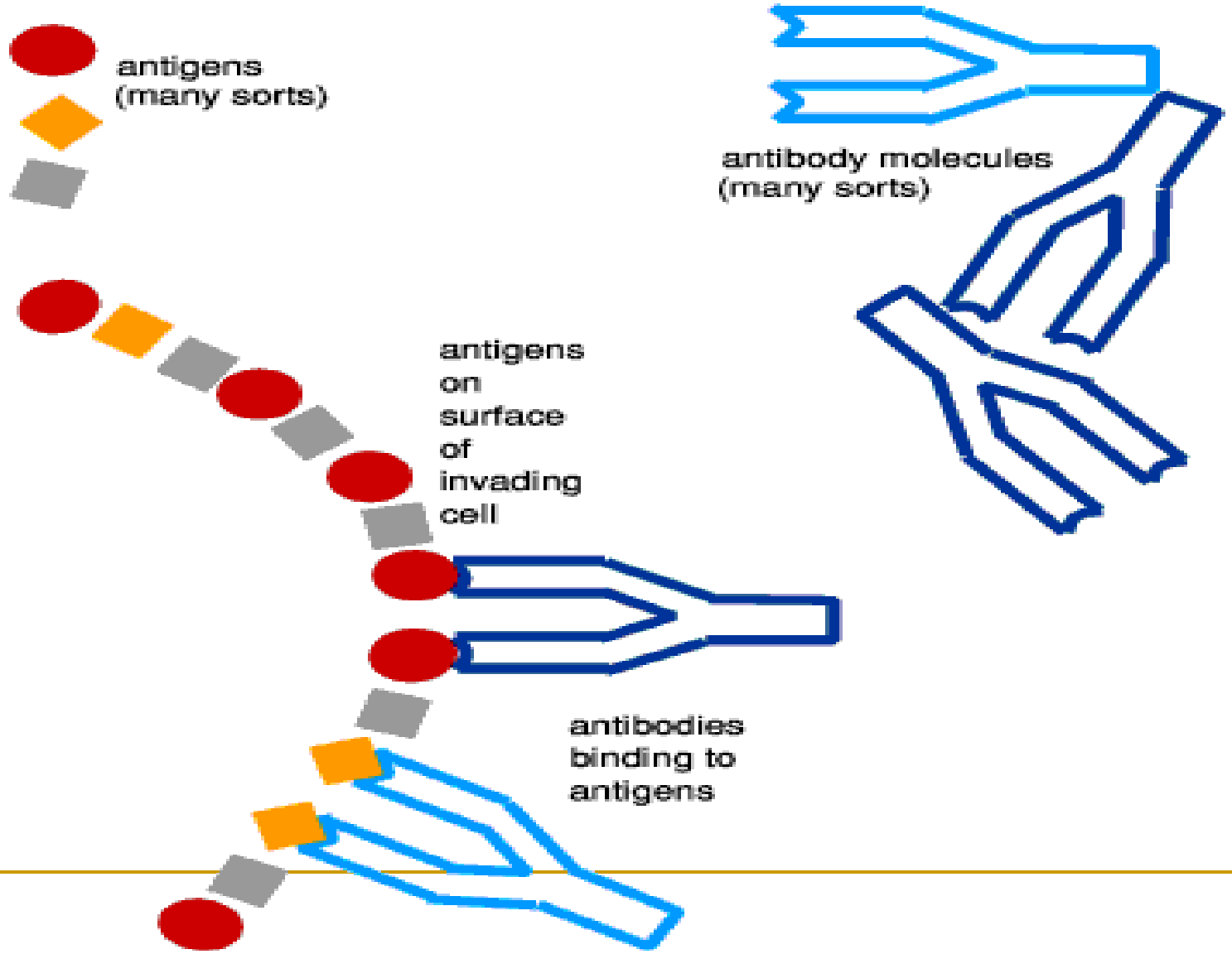
- **There are over 300 known blood group antigens**
- **>10,00,000 different antigen sites on each RBC**
- **Antigens are attached to proteins or lipids on the RBC membrane and are usually complex sugar groups**
- **Some stick out far on RBC membrane and some are buried within crypts on the membrane surface**

Antibody

- **Proteins produced by immune system as a result of stimulation by an antigen which can then interact specifically with that particular antigen.**
- **Immunoglobulin:**
- **Important classes of Abs in BB: IgM & IgG,**



The antigen-antibody reaction



Rules of Ag-Ab reactions

- **If a person's cell have the Ag, the Ab should NOT be present in that person's serum**
- **If an Ab to a blood group Ag is present in the serum of a person, his or her cells should lack that antigen**
- **The Ags are on the cells and the Abs are in the serum**
- **Combination of Ag-Ab produce variety of observable results, most common are agglutination, hemolysis, precipitation**

Ag Ab reactions (1)

1. Agglutination

Ab mediated clumping of particles that express Ag on surface, clumping occurs because Ab molecules binds to Ag determinants on red cells brining them together into visible aggregates

2. Precipitation

Formation of insoluble visible complex, seen as sediments

Ag Ab reactions (2)

3. Hemolysis

Rupture of red cells with release of Hb

In vitro hemolysis depends on MAC

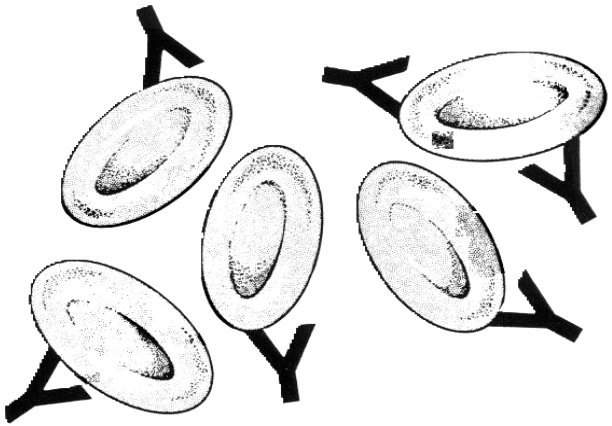
Hemolysis is positive reaction, pink or red supernatant fluid

4. Inhibition

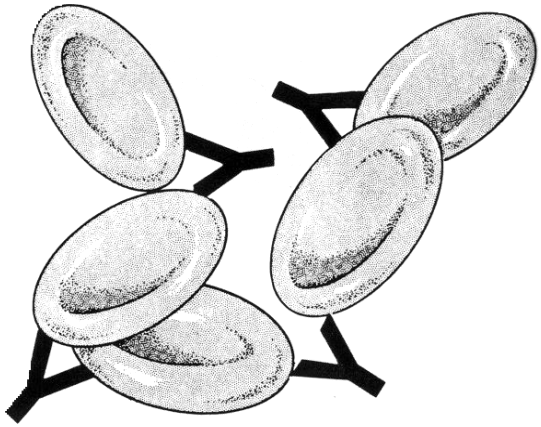
Presence of Ag-Ab is detected by inhibition of previously documented agglutination (eg. saliva test)

Stages of Ag-Ab Interaction

- ***First stage is sensitization:*** **when antibodies react with antigens on the cells and coat the cells.**

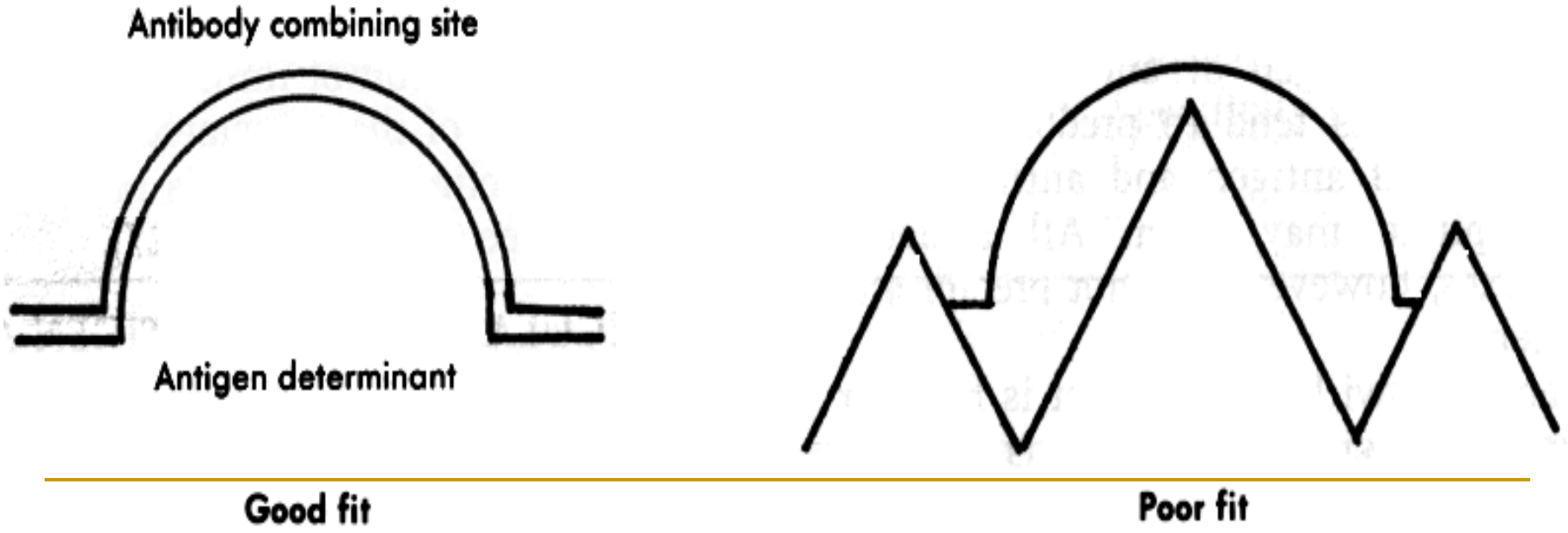


- ***Second stage is agglutination:*** **when antibodies on coated cells form cross-linkages b/w cells resulting in visible clumping.**



First stage of agglutination (1)

- **Ag Ab come together**
- **Specificity depends on the spatial and chemical "fit" between antigen and antibody**



First stage of agglutination (2)

Chemical bonding

1. **Polar bonding-electrons are interchanged, reactions taking place in water based medium**
2. **Hydrophobic bonds- interactions b/w non polar molecules**
3. **Van der Waals bonds results form mutual attraction b/w all molecules when brought closer**
3. **Electrostatic or ionic bonds- attraction of ionized or oppositely charged molecules**

First stage of agglutination (3)

- **Equilibrium constant (K_0) –relative rates of association and dissociation.**
- **For each Ag Ab reaction K_0 varies**
- **K_0 reflects degree of fit & speed, higher the K_0 better the association/fit**
- **K_0 large–difficult to dissociate; high clinical importance**
- **K_0 small, high ratio of Ag to Ab required**
- **K_0 affected by physical conditions- temperature, pH & ionic strength of medium, relative Ag Ab concentration**

First stage of agglutination (4)

Temperature:

- Most blood group Ab react within restricted temp range
- Two categories: cold reacting (4-25^0 c)
  : warm reactive (30-37^0 c)
- Ab reacting <37^0 c: clinically insignificant

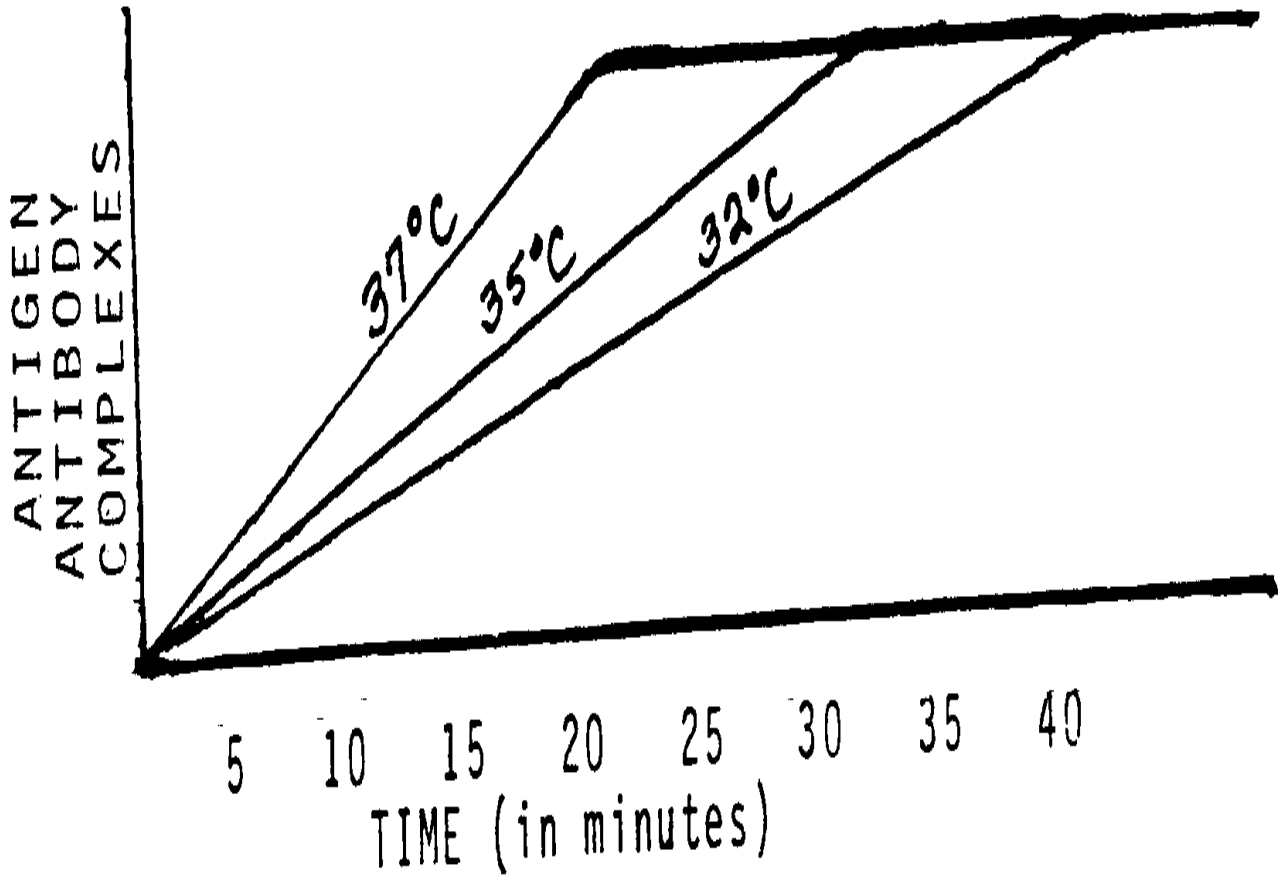
Carbohydrates Ag: more cold reactive

Proteins Ag: warm reactive

First stage of agglutination (5)

Temperature

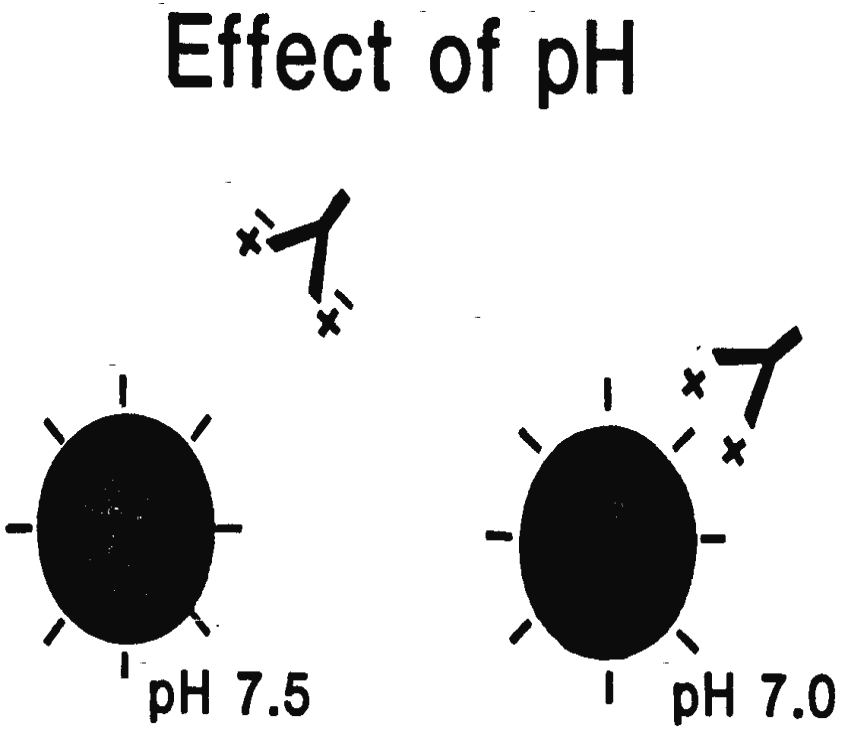
- IgG best at 37ºC;



First stage of agglutination (6)

pH:

- Change in pH affect non-covalent & electrostatic bonds
- Optimum pH physiologic ranges-7.0



First stage of agglutination (7)

Incubation time:

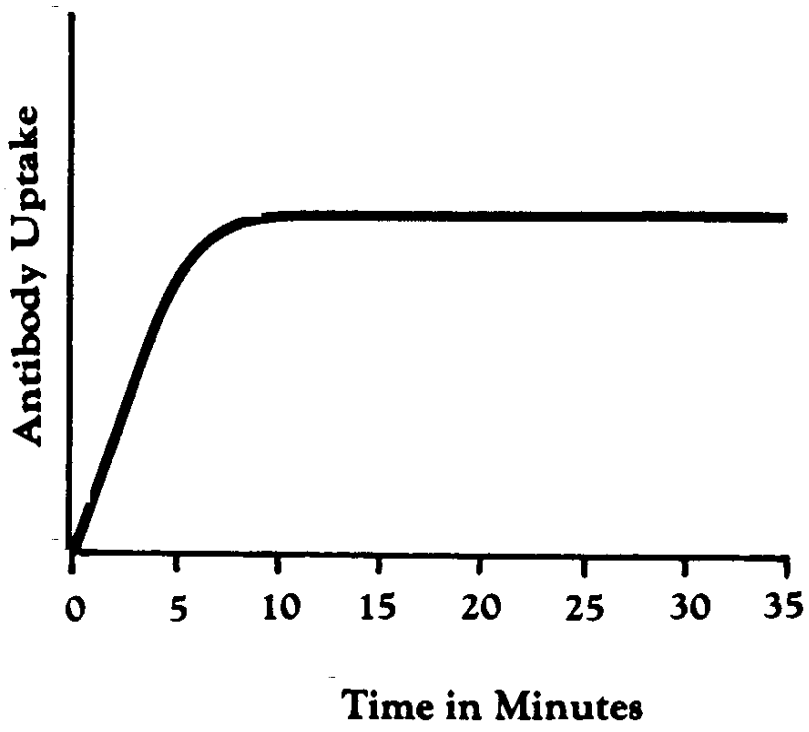
- Time needed to reach equilibrium differs for different bld grp Ab
- Variables are temp, Ig class, specific interaction b/w Ag configuration
- Saline system: 30-60 minutes incubation is adequate
- In LISS –time reduced to 10-15 minutes

First stage of agglutination (8)

Incubation time

- Ag accessibility is also important. ABO Ags, are on the surface of the red cell while others may be hidden in the crypts of the cell membrane.

- Enhancement agents can ↓ the incubation time



First stage of agglutination (9)

Ionic Strength:

- In normal saline Na^+ & Cl^- cluster around and partially neutralize opposite charges on Ag & Ab molecules which hinders the association of Ab with Ag

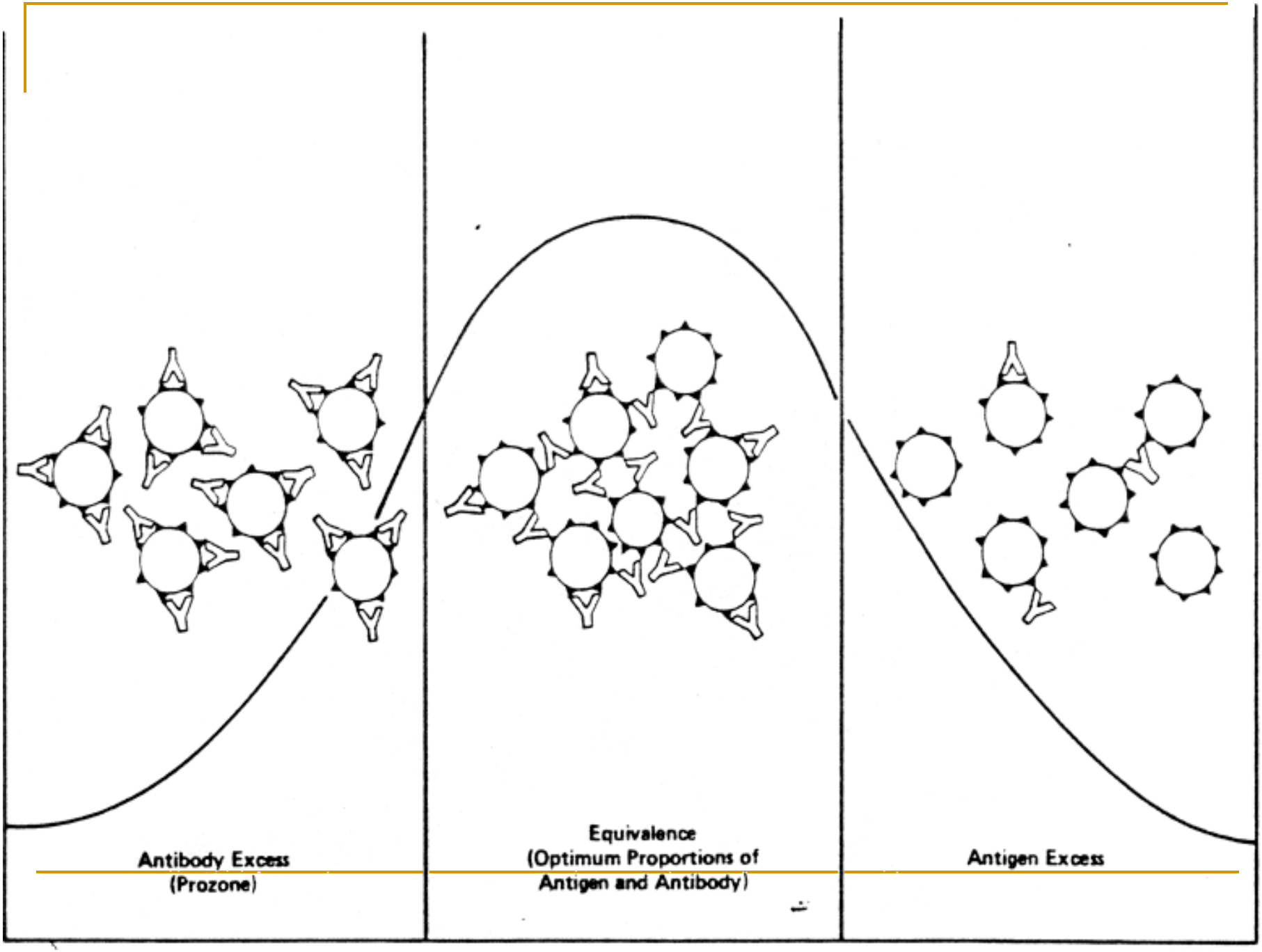
- By lowering ionic strength this shielding effect weakened & the electrostatic attraction enhanced

First stage of agglutination (10)

Ag- Ab proportion

- Prozone - antibody excess: Abs saturating all Ag sites; no cross-linkages between cells; no agglutination
- Zone of equivalence: Abs & Ags present in optimum ratio, agglutination formed
- Postzone- antigen excess

too many Ags - any agglutination

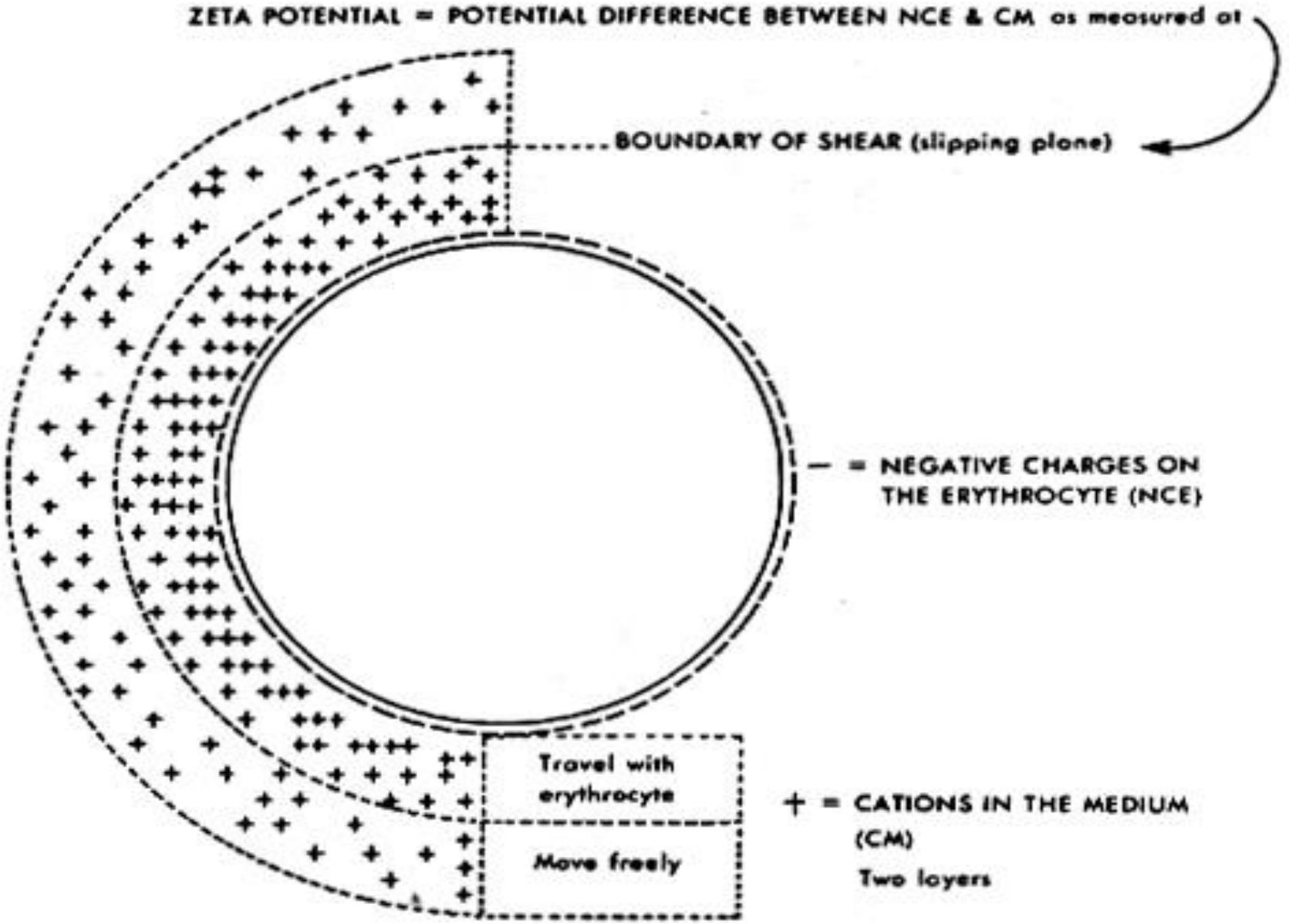

Antibody Excess
(Prozone)
Equivalence
(Optimum Proportions of
Antigen and Antibody)
Antigen Excess

Second stage of Agglutination (1)

- The sensitized linked into lattice- allows visualization of reaction
- Size & physical properties, concentration of Ag site on each cell, distance b/w cells
- IgG molecules fails to bridge this distance & cause sensitization without lattice formation, for Ig M direct agglutination occurs easily
- Location & density of Ag allow some IgG to cause direct agglutinate e g. A B M N are on outer edge of RBC

Second stage of Agglutination (2)

- **Red cells in saline have net negative charge due to high sialic acid**
- **Red cells suspended in ionic medium cations arranges themselves around red cells to form ionic cloud; cations closest to cell are firmly bound & move with red cell while the outer cloud move freely**
- **The difference in charge at surface b/w inner & outer layer is surface of shear creates an electrical potential called zeta potential**
- **Distance b/w red cells in ionic medium is proportional to zeta potential**


ZETA POTENTIAL = POTENTIAL DIFFERENCE BETWEEN NCE & CM as measured at
BOUNDARY OF SHEAR (slipping plane)
— = NEGATIVE CHARGES ON THE ERYTHROCYTE (NCE)
Travel with erythrocyte
Move freely
+ = CATIONS IN THE MEDIUM (CM)
Two layers

Second stage of Agglutination (3)

Various strategies to enhance second-stage of agglutination

- Centrifugation force the cells together
- Reducing negative charge, reducing hydration layer

Antibody enhancement methods (1)

Albumin additives

- Albumin influence the second stage by reducing the net negative charge

- Bovine serum albumin is available as 22% & as polymerized solution

Antibody enhancement methods (2)

Enzymes

- Bromelin, ficin, papain & trypsin
- Proteolytic enzyme reduces the surface charge by cleaving sialic acid from polypeptide chain
- Enzyme lower the zeta potential, reduces polar repulsion & increases interfacial tension, causes spicule formation –increases contact points

Antibody enhancement methods (3)

Positively charged Molecules

- In presence of positively charged polymers like polybrene, protamine sulfate, poly-L-lysine red cells exhibits spontaneous aggregation

Polyethylene Glycol (PEG)

- PEG is water soluble linear polymer- increase Ab uptake
- Action is to remove water

Antibody enhancement methods (4)

LISS

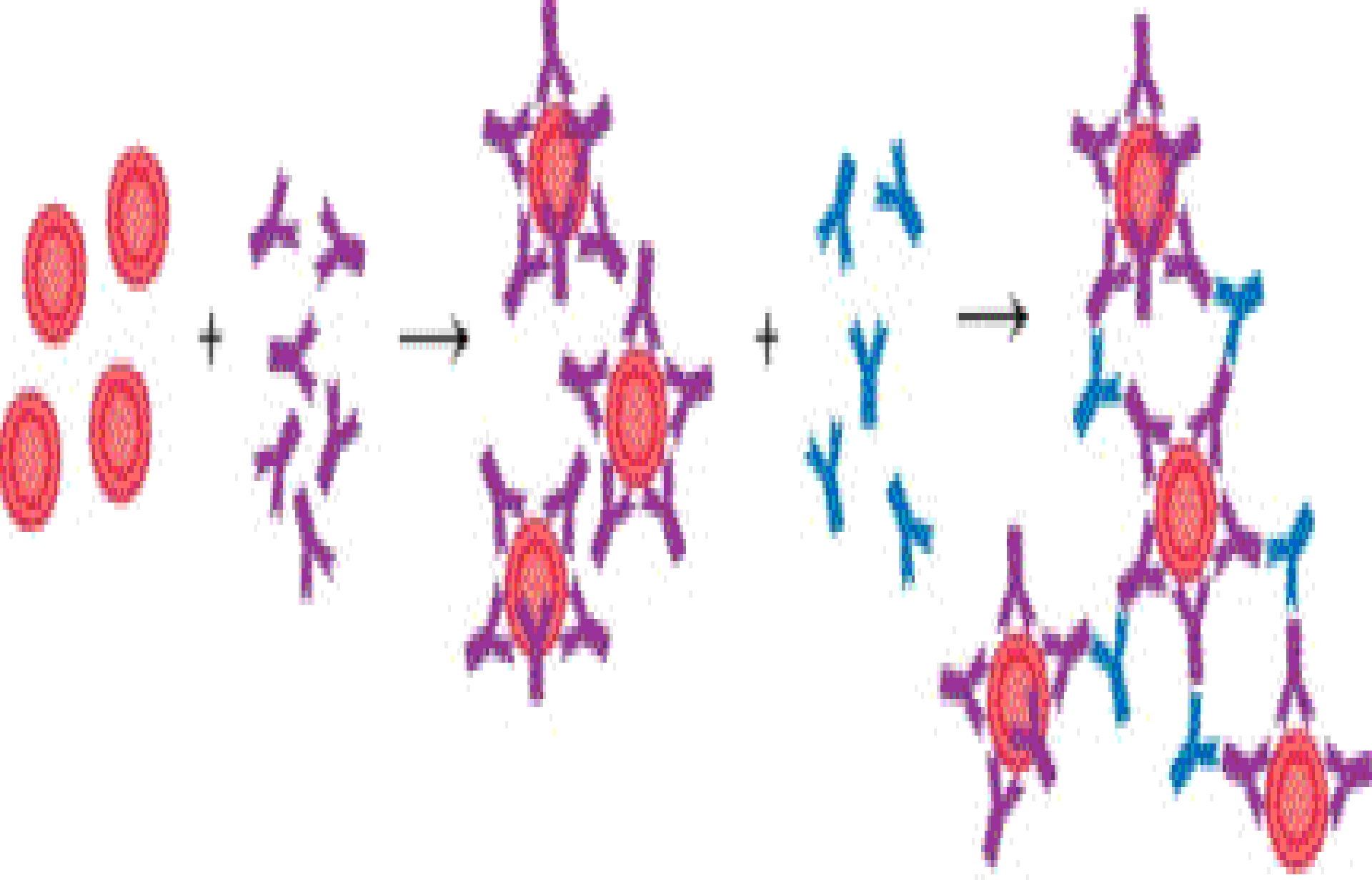
- LISS increases first stage, to prevent lysis glycine is incorporated in LISS
- LISS additive is more commonly used
- Commercially available
- While using LISS manufacturer’s instructions must be followed

Antihuman globulin test

Coombs' test

- Principle:

  The AHG acts as bridge and induces agglutination of sensitized red cells
- DAT : to demonstrate in vivo sensitization
- IAT: to demonstrate in vitro sensitization


Antigen
Antibody
No
Anti-Ig
Agglutination

Other Methods (1)

Column agglutination, Gel tests

- **microtubes, space at top**
- **as the cells passes thru the column medium separates agglutinated from unagglutinated cells**
- **negative test cells pellet to bottom, positive results- the cells are captured at top or within column**

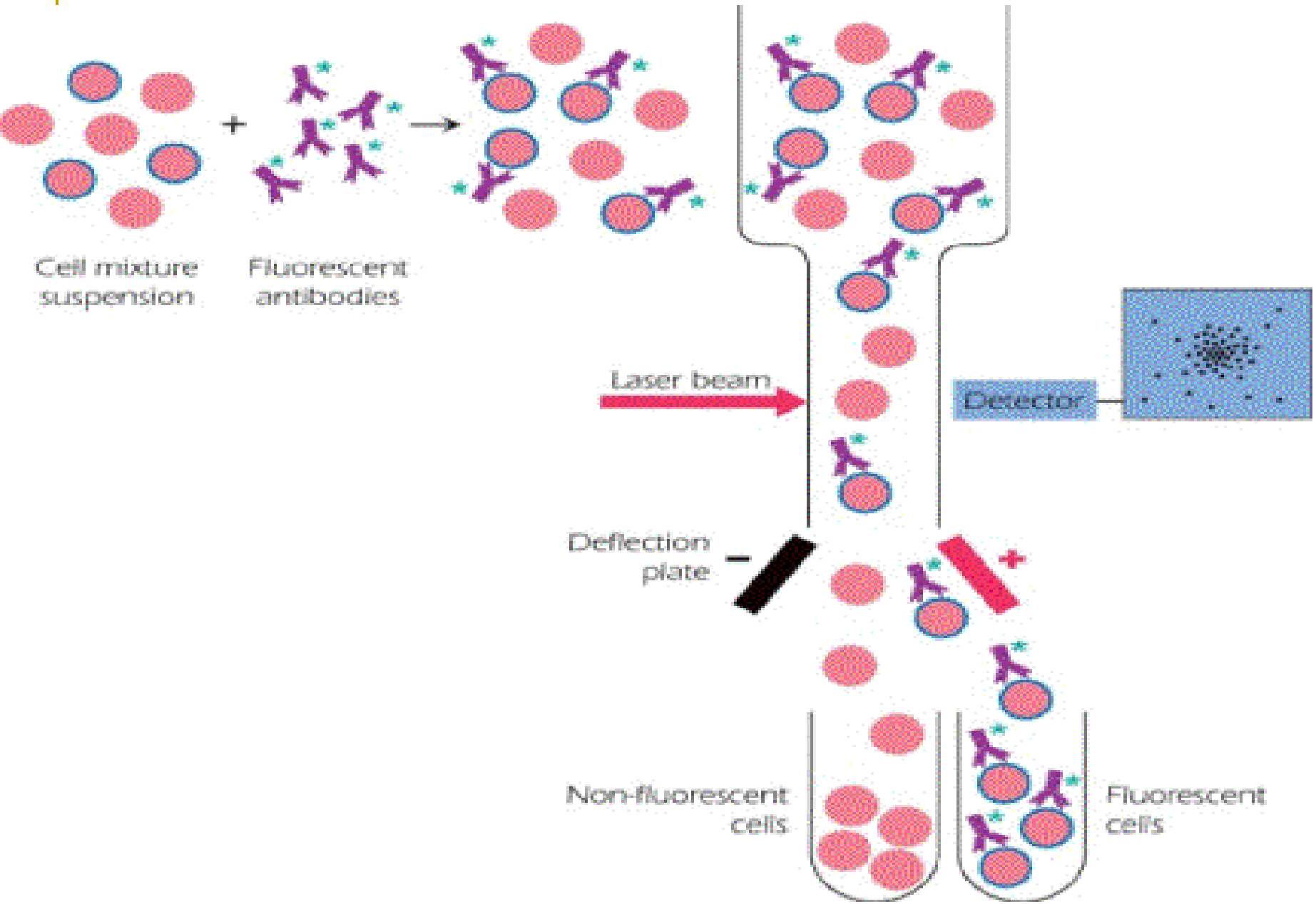
Solid Phase red cells adherence tests

- **Utilize immobilized Ag or Ab**

Other Methods (2)

Immunofluorescence

- **Allows identification & localization of Ag inside or on surface of cells**
- **Flurochrome (fluorescein or phycoerythrin) can be attached on Ab without affecting its specificity & ability**
- **Attachment of fluorescein labeled Ab to Ag makes Ab coated cells appears fluroscent**
- **Can be direct or indirect**
- **Used in flow cytometry**


Cell mixture suspension
Fluorescent antibodies
Laser beam
Detector
Deflection plate
Non-fluorescent cells
Fluorescent cells

Other Methods (3)

Radioimmunoassay

- **use radionucleotide as a marker**
- **radiolabeling does not affect Ab specificity**

ELISA

- **used to measure Ag or Ab**
- **enzymes are used as label, being more safer, cheaper, & simpler to measure**

Other Methods (4)

MAIEA (Monoclonal antibody specific immobilization of erythrocyte antigens)

- **Red cells -incubated with 2 Abs-one contains human alloAb to blood group & other is nonhuman Ab which react with different portion of membrane protein**
- **Red cells lysed & solubilized- added to microwell plate coated with goat antimouse Ab**
- **Reaction is ELISA readable**
- **Used to isolate specific membrane structure for blood group Ag**

Thank You