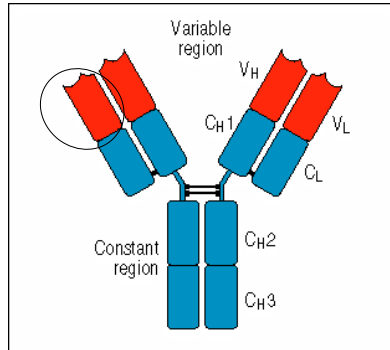


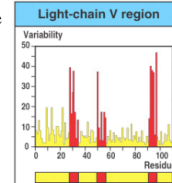
## A Brief Review of Antibody Structure

The basic antibody is a dimer of dimer (2 heavy chain-light chain pairs) composed of repeats of a single structural unit known as the “immunoglobulin domain”

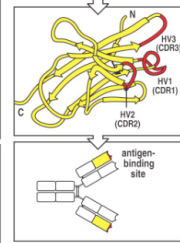


## A Brief Review of Antibody Structure

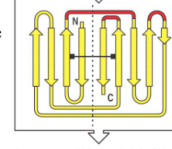
Primary structure



Tertiary structure



Secondary structure



Quaternary structure

Figure 3-7 Immunobiology 6/e. © Garland Science 2005

## Antigens & Antibodies II

Definitions

A comparison of antigen recognition by B and T cells

Factors that influence immunogenicity

### Quantitating the strength of antibody-antigen interactions

Equilibrium constants

equilibrium dialysis

impact of multivalency

Cross-reactivity of antibodies

Measuring antibody-antigen binding

## Polyclonal antibodies vs Monoclonal antibodies

**Polyclonal antibodies:** antibody preparations from immunized animals. Consist of complex mixtures of different antibodies produced by many different B cell clones

**Monoclonal Antibody:** homogeneous antibody preparations produced in the laboratory. Consist of a single type of antigen binding site, produced by a single B cell clone (later we'll talk about how these are made).

## Scatchard analysis

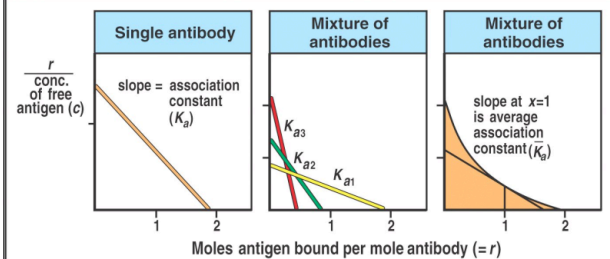
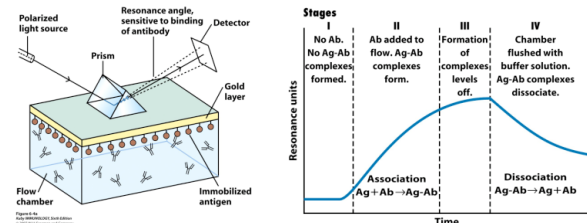


Figure A-11 part 2 of 2 Immunobiology, 6/e. © Garland Science 2005

## Affinity between two macromolecules can be measured using a biosensor

Technique: Surface Plasmon Resonance

Instrument: Biocore



-Resonance units are proportional to the degree of binding of soluble ligand to the immobilized receptor. (or soluble antibody to immobilized antigen, as shown here)  
- Determining the amount of binding at equilibrium with different known concentrations of receptor (antibody) and ligand (protein antigen) allows you to calculate equilibrium constants ( $K_a$ ,  $K_d$ ).

-Rate of dissociation and association ( $k_{off}$ ,  $k_{on}$ ) can also be calculated.

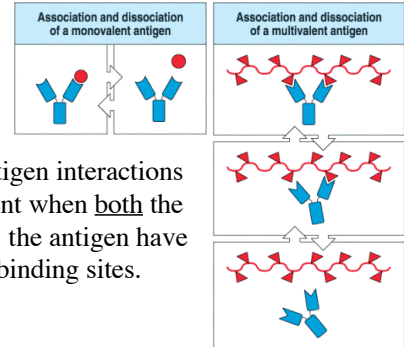
Affinity refers to strength of binding of single epitope to single antigen binding site.

But antibodies have 2 or more identical binding sites.

Most antigens are multivalent.

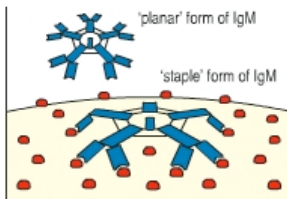
What is impact of valence on strength of binding?

Avidity (strength of binding) is influenced by both Affinity ( $K_a$  of single binding site)  $\times$  Valence of interaction (number of interacting binding sites)



Antibody-antigen interactions are multivalent when both the antibody and the antigen have multiple binding sites.

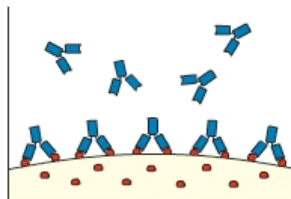
### Decavalent IgM



low affinity interactions can have high avidity if valence is high.

IgM tend to bind tightly, but have less specificity.

### Bivalent IgG



Avid binding due to high affinity. Binding of IgG tends to be more specific. (more perfect "fit" between antigen binding site and antigen)

## Antigens & Antibodies II

Definitions and comparison of B and T cell antigen recognition

Quantitating the strength of antibody-antigen interactions: affinity and avidity

- Equilibrium constants
- equilibrium dialysis
- impact of multivalency

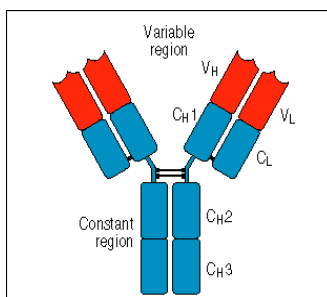
### Cross-reactivity of antibodies

#### Definition of cross-reactivity

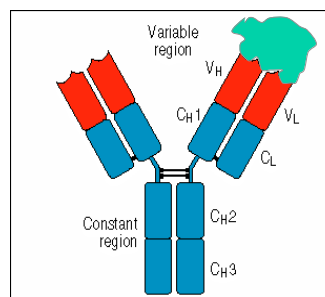
- Example: ABO blood groups
- Cause of rheumatic fever (streptolysin)
- Useful for vaccine generation
- Problems for self-tolerance

Measuring antibody-antigen binding

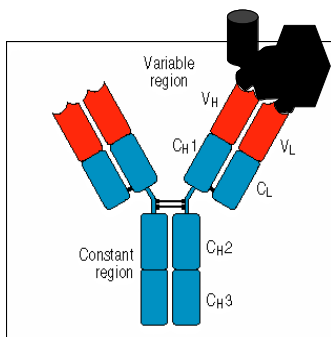
## Cross-reactive Antigens



## Cross-reactive Antigens



# Cross-reactive Antigens



# Antigens & Antibodies II

Definitions and comparison of B and T cell antigen recognition

Quantitating the strength of antibody-antigen interactions: affinity and avidity

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### Cross-reactivity of antibodies

- Definition of cross-reactivity
- Example: ABO blood groups**
- Cause of rheumatic fever (streptolysin)
- Useful for vaccine generation
- Problems for self-tolerance

Measuring antibody-antigen binding

ABO blood types are due to presence of antibodies that cross-react with polysaccharide antigens on red blood cells

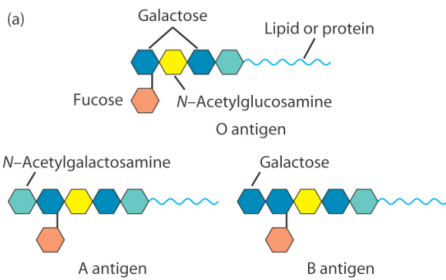


TABLE 6-2 ABO blood types

Blood type	Antigens on RBCs	Serum antibodies
A	A	Anti-B
B	B	Anti-A
AB	A and B	Neither
O	Neither	Anti-A and anti-B

Antibodies are induced by microbial antigens, but cross react with polysaccharide antigens found on red blood cells.

Individual will not produce antibodies that react with own RBC (self-tolerance).

The blood type of an individual can be determined by an agglutination assay: look for ability of serum to bind to, and agglutinate RBC from another individual.

		Red blood cells from individuals of type			
		O	A	B	AB
		Express the carbohydrate structures			
Serum from individuals of type		R-GlcNAc-Gal-Fuc	R-GlcNAc-Gal-GalNAc-Fuc	R-GlcNAc-Gal-Fuc	R-GlcNAc-Gal-GalNAc-Fuc R-GlcNAc-Gal-Fuc
universal donor	O Anti-A and anti-B antibodies	no agglutination	agglutination	agglutination	agglutination
	A Anti-B antibodies	no agglutination	no agglutination	agglutination	agglutination
	B Anti-A antibodies	no agglutination	agglutination	no agglutination	agglutination
universal recipient	AB No antibodies to A or B	no agglutination	no agglutination	no agglutination	no agglutination

Figure 4-8 Immunobiology, 6/e. (© Garland Science 2005)

		Red blood cells from individuals of type			
		O	A	B	AB
		Express the carbohydrate structures			
Serum from individuals of type		R-GlcNAc-Gal-Fuc	R-GlcNAc-Gal-GalNAc-Fuc	R-GlcNAc-Gal-Fuc	R-GlcNAc-Gal-GalNAc-Fuc R-GlcNAc-Gal-Fuc
Anti-A and anti-B antibodies	O	no agglutination	agglutination	agglutination	agglutination
Anti-A antibodies	A	no agglutination	no agglutination	agglutination	agglutination
Anti-B antibodies	B	no agglutination	agglutination	no agglutination	agglutination
No antibodies to A or B	AB	no agglutination	no agglutination	no agglutination	no agglutination

Figure 4-8 Immunobiology, 6/e. (© Garland Science 2005)

Why do A individuals have antibodies against B type RBC carbohydrates?

Why don't A individuals have antibodies against A type RBC carbohydrates?

## Antigens & Antibodies II

Definitions and comparison of B and T cell antigen recognition

Quantitating the strength of antibody-antigen interactions: affinity and avidity

- Equilibrium constants
- equilibrium dialysis
- impact of multivalency

### Cross-reactivity of antibodies

- Definition of cross-reactivity
- Example: ABO blood groups
- Cause of rheumatic fever (streptolysin)**
- Useful for vaccine generation
- Problems for self-tolerance

Measuring antibody-antigen binding

## Rheumatic Fever

- Complication arising from infection with *Streptococcus pyogenes*
- antibodies to bacterial proteins (M antigen or streptolysin) cross-react with myocardial and muscle proteins.

## Taking advantage of cross-reactivity in vaccine design

- Immunization with cowpox (vaccinia virus) induces immunity to smallpox (variola virus). (Jenner)
- Vaccination to one type of influenza virus provides resistance to other forms of influenza.

## Cross-reactivity and self-tolerance

- Our bodies contain many epitopes that resemble the epitopes found on pathogens.
- By avoiding reactivity to those self-antigens, we restrict the ability of our immune systems to recognize certain pathogens.
- Tolerance to polysaccharide antigens on RBC prevents the production of certain antibodies reactive to microbial antigens.

## Antigens & Antibodies II

Definitions and comparison of B and T cell antigen recognition

Quantitating the strength of antibody-antigen interactions: affinity and avidity

- Equilibrium constants
- equilibrium dialysis

Cross-reactivity of antibodies

### Measuring antibody-antigen binding

- lattice formation and precipitation reactions**
- secondary antibodies: antibodies reactive with other antibodies
- medical tests based on antibody-antigen precipitation reactions

Assay	Sensitivity* ( $\mu\text{g antibody/ml}$ )
Precipitation reaction in fluids	20–200
Precipitation reactions in gels	
Mancini radial immunodiffusion	10–50
Ouchterlony double immunodiffusion	20–200
Immunoelectrophoresis	20–200
Rocket electrophoresis	2
Agglutination reactions	
Direct	0.3
Passive agglutination	0.006–0.06
Agglutination inhibition	0.006–0.06
Radiolimmunoassay (RIA)	0.0006–0.006
Enzyme-linked immunosorbent assay (ELISA)	–0.0001–0.01
ELISA using chemiluminescence	–0.00001–0.01 <sup>†</sup>
Immunofluorescence	1.0
Flow cytometry	0.006–0.06

\*The sensitivity depends on the affinity of the antibody used for the assay as well as the epitope density and distribution on the antigen.  
<sup>†</sup>Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.  
 SOURCE: Updated and adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology*, 5th ed., American Society for Microbiology, Washington, DC.

A variety of different assays have been developed to detect antibody-antigen interactions.

Some of these are based on the tendency of antibody-antigen complexes to come out of solution called “precipitation reaction”.

Some are based on the ability of antibodies to stick cells together, called an “agglutination reaction”.

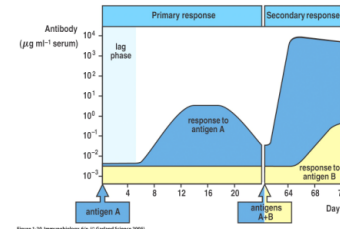
## Precipitation Reactions:

Antibody and Antigen interactions in solution can lead to the formation of a lattice and precipitation of immune complexes.

Antibody and antigen must be multivalent.

Occurs most efficiently when antigen and antibody are at similar concentration.

## Polyclonal antisera



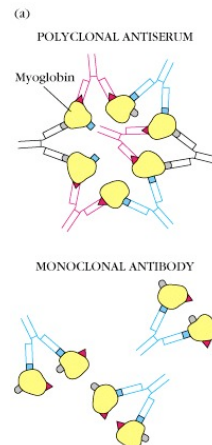
Can be generated by repeated immunization of animal (rabbit) with antigen (with adjuvant).

polyclonal antibodies are a complex mixture of antibodies directed against different epitopes and that differ in their affinity for the antigen.

## Polyclonal antibodies vs Monoclonal antibodies

Polyclonal antibodies: antibody preparations from immunized animals. Consist of complex mixtures of different antibodies produced by many different B cell clones

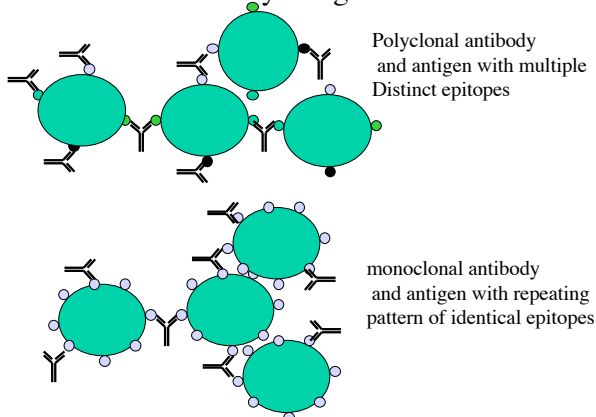
Monoclonal Antibody: homogeneous antibody preparations produced in the laboratory. Consist of a single type of antigen binding site, produced by a single B cell clone (later we'll talk about how these are made).



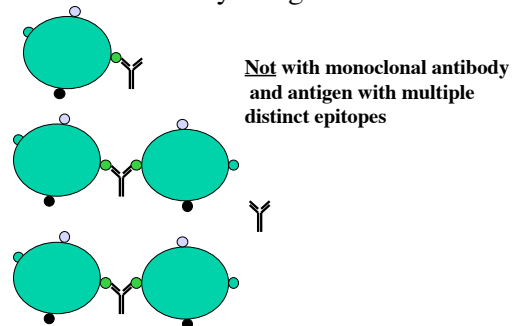
Polyclonal antibodies can form lattices with homogeneous, monomeric protein antigens because each antibody can interact with a different epitope on the antigen.

Monoclonal antibodies do not form lattices with homogeneous, monomeric proteins, because only they can bind to only one epitope on the antigen.

## When do antibody-antigen lattices form?




## When do antibody-antigen lattices form?

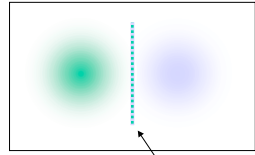


## Double-diffusion assay (Ouchterlony method)

antigen    antibody



Antigen & antibody  
embedded in agar  
gel atop glass slide



Line of precipitation

## DOUBLE IMMUNODIFFUSION

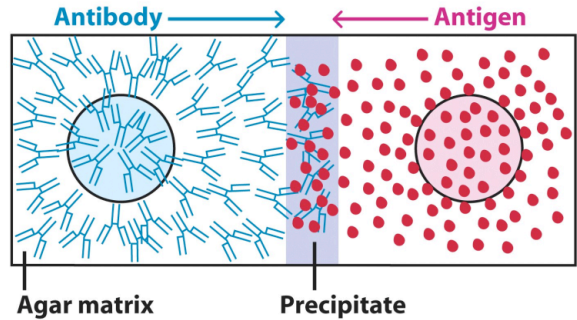
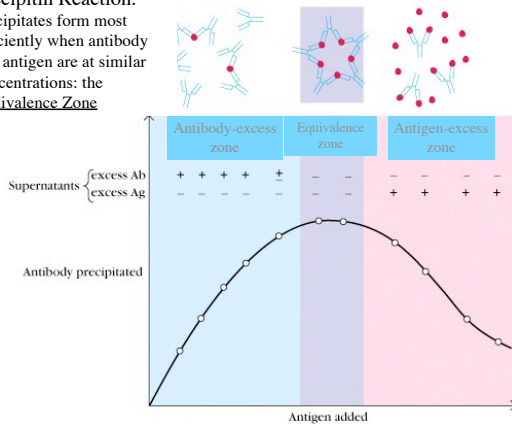


Figure 6-6 part 2  
Kuby IMMUNOLOGY, Sixth Edition  
© 2007 W. H. Freeman and Company

Precipitin Reaction:  
precipitates form most  
efficiently when antibody  
and antigen are at similar  
concentrations: the  
Equivalence Zone



Antibody binding to cells can bind cells together  
into a large clump: an “agglutination reaction”

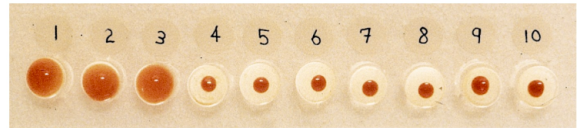


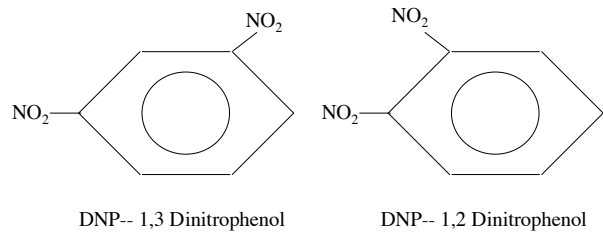
Figure 6-8  
Kuby IMMUNOLOGY, Sixth Edition  
© 2007 W. H. Freeman and Company

Wells contain sheep red blood cells (SRBC) and  
increasing concentration of anti-SRBC antibodies

Agglutination reaction based on  
antibody binding to the hormone  
human chorionic gonadotrophic  
hormone (HCG).

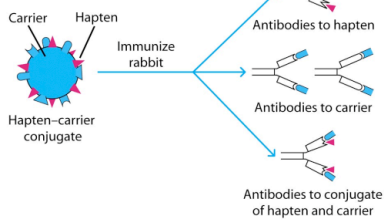
But first, a brief reminder about  
haptens and carriers. . .

**Hapten:** ANY small molecule that cannot by itself  
induce an immune response, but can be an antigen.



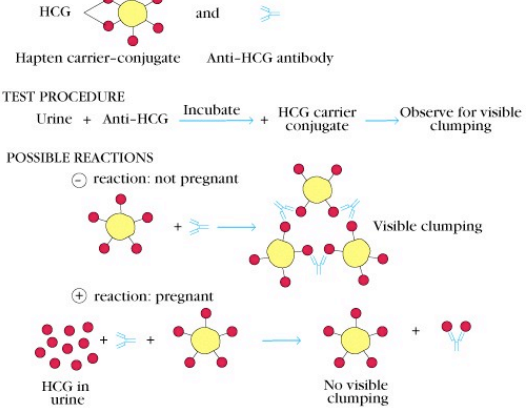
Even closely related haptens can be distinguished antigenically;  
antibodies raised against 1,2 DNP may not react with 1,3 DNP.

Haptens are not immunogenic unless they are coupled to a carrier protein.



Injection with:	Antibodies formed:
Hapten (DNP)	None
Protein carrier (BSA)	Anti-BSA
Hapten-carrier conjugate (DNP-BSA)	Anti-DNP (major)
	Anti-BSA (minor)
	Anti-DNP/BSA (minor)

### Agglutination-based pregnancy test



## Antigens & Antibodies II

Definitions and comparison of B and T cell antigen recognition

Quantitating the strength of antibody-antigen interactions: affinity and avidity

Equilibrium constants  
equilibrium dialysis

Cross-reactivity of antibodies

Measuring antibody-antigen binding

lattice formation and precipitation reactions

**secondary antibodies: antibodies reactive with other antibodies**

medical tests based on antibody-antigen precipitation reactions

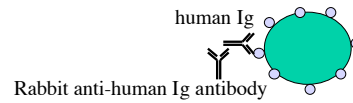
## Antibodies that bind to other antibodies (secondary antibodies)

Immunize animal (rabbit) with purified antibodies from another species (human).

The human Ig is the antigen, and the antibodies raised in the rabbit bind specifically to human Ig.

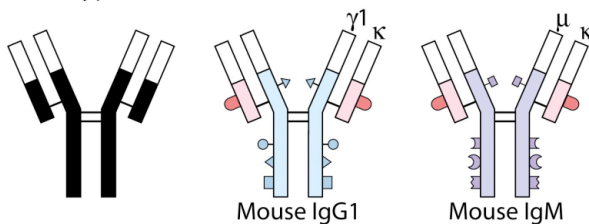
These anti-human Ig (secondary antibodies) can be used to detect presence of human Ig.

Use of anti-Ig antibodies increases degree of cross-linking and can increase lattice formation.



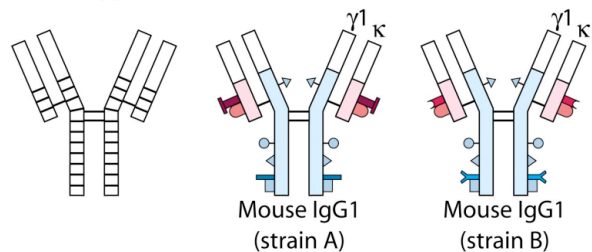
Some secondary antibodies recognize portions of the constant regions that are characteristic of a particular antibody isotype. (These are the most common types of secondary antibodies.)

### (a) Isotypic determinants



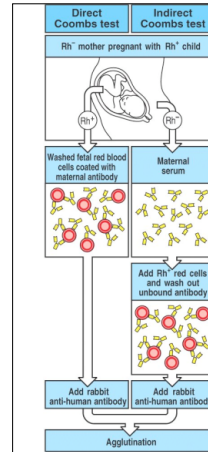
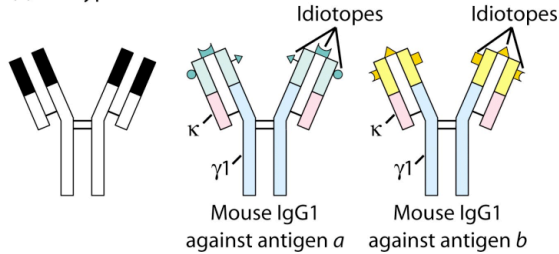
Some secondary antibodies recognize portions of the antibody that are variable between different individuals (different allotypes) in the species.

### (b) Allotypic determinants



Some secondary antibodies recognize unique portions of the variable domain of the antibody: the antigen binding sites (idiotypes). Anti-idiotypic antibodies are rare.

(c) Idiotypic determinants



Agglutination as a clinical assay-- Testing for Rh incompatibility

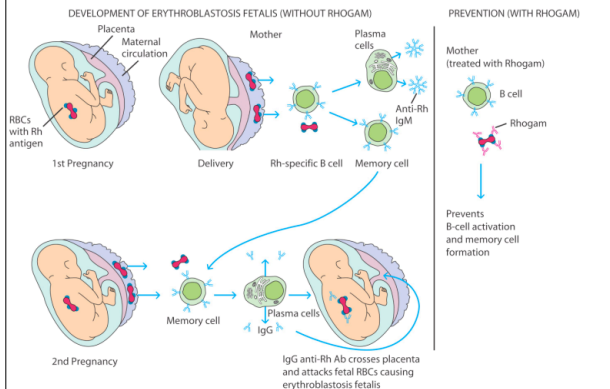
**Disease:**  
Erythroblastosis fetalis

**Cause:**  
Mother produces IgG that bind to an antigen (Rh) on RBC of fetus

**Detection:**  
Expose RBC to anti-human Ab and look for agglutination

Figure A-13 Immunobiology, 6/e. © Garland Science 2005

Treatment of mothers with antibodies to Rh (RhoGam) at time of 1st delivery can prevent her from developing anti-Rh antibodies.



## Immunological Techniques

### Monoclonal Antibodies

- Radioimmune Assay (RIA)
- Enzyme Linked Immune Sorbant Assay (ELISA)
- Western blot
- Immunoprecipitation
- Flow cytometry
- Expression cloning

## Limitations of polyclonal antisera

Polyclonal antibodies are a complex mixture of antibodies directed against different epitopes and that differ in their affinity for the antigen.

Each antisera preparation differs in specificity, average affinity, cross-reactive specificities, etc.

Supply is limited.

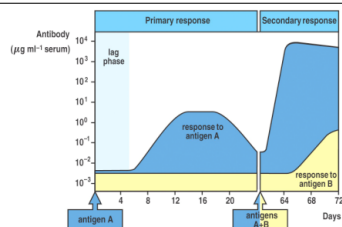


Figure 1-28 Immunobiology, 6/e. © Garland Science 2005

## Monoclonal antibodies

Kohler and Milstein, 1975

a technique to generate inexhaustible supply of homogeneous antibody with useful specificities.

Basic strategy: fuse 2 cell types to generate a "hybridoma". Takes advantage of the properties of myeloma cell (unlimited growth capacity and cellular machinery to produce antibodies) and the antigen specificity of primary B cells.



## What is a clone?

A population of cells derived from a single progenitor cell.

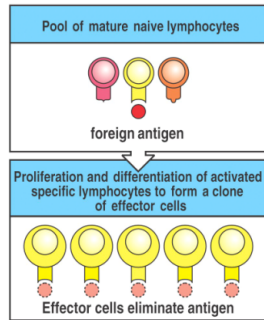
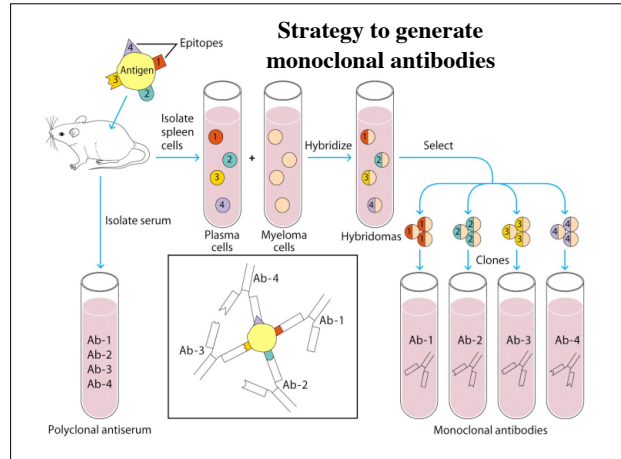


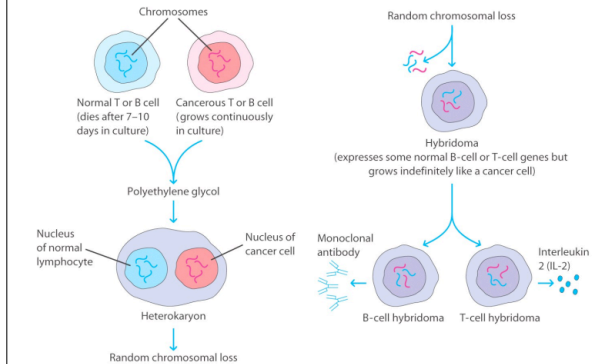
Figure 1-14 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)

The adaptive immune system works by **clonal selection**. Antigen triggers the expansion of **limited number of clones** that are specific for that particular antigen.

## Strategy to generate monoclonal antibodies



Hybridomas are hybrids between a non-transformed antibody producing B cell and a transformed cell (myeloma) that can grow continuously in culture.



HAT selection is used to select for growth of hybrids and against the growth of the parental myeloma.

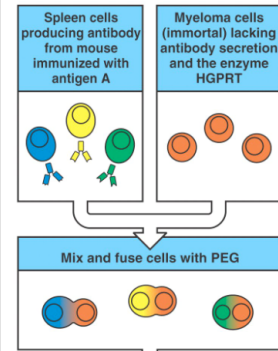


Figure A-14 part 1 of 2 Immunobiology, 6/e. (© Garland Science 2005)

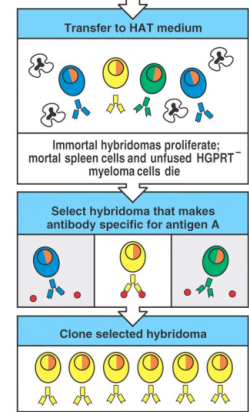
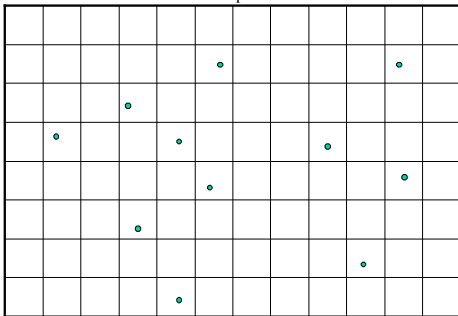


Figure A-14 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)

## Cloning hybridomas from fusion

Plate at limiting dilution (<1 cell/well) in 96 well plates.  
Allow clones to expand.

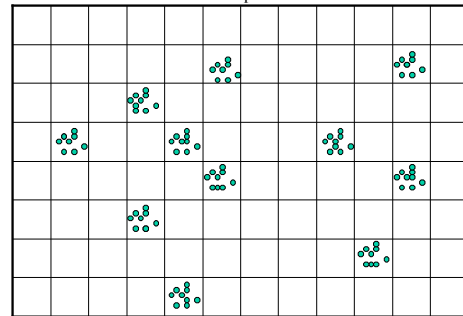
Expand positive well and test for production of antibody of desired specificity in culture supernatant



## Cloning hybridomas from fusion

Plate at limiting dilution (<1 cell/well) in 96 well plates.  
Allow clones to expand.

Expand positive well and test for production of antibody of desired specificity in culture supernatant



## Advantages of Monoclonal Abs

- Consistent
- Limitless supply of specific reagent
- More easily tested for cross-reactivity