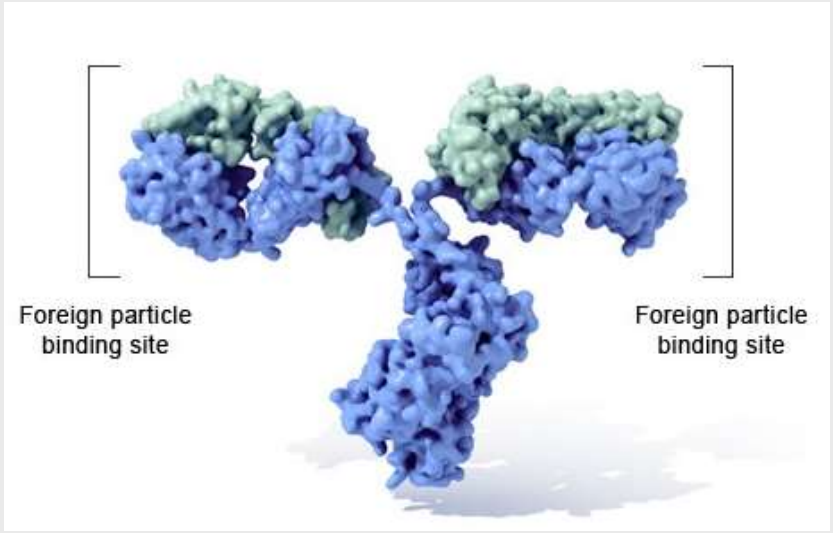
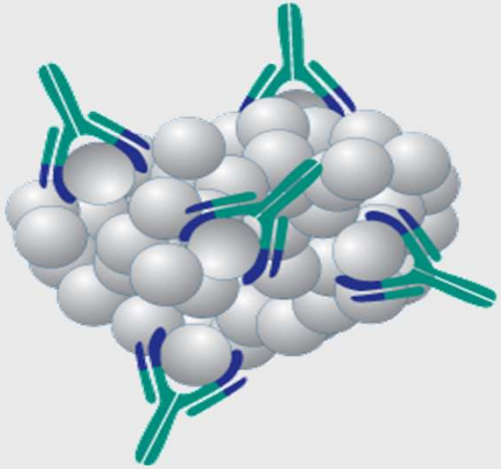
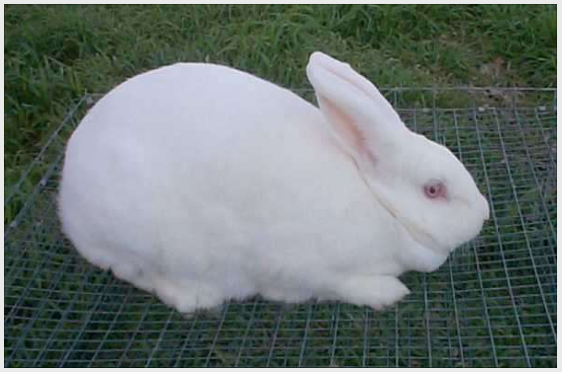
Serology and Plant Viruses







Objectives:

- Be able to describe an antibody and how one is produced
- Be able to describe an antigen and how an antigen is produced (plant virology)
- Understand the structure of antibodies and how that relates to their use in detection assays
- Understand the similarities and differences of the 2 basic types of antibodies
- Understand how antibodies and antigens bind, and how we exploit that interaction for virus (protein) detection

Serology: the science dealing with the reactions and characteristics of serum

The Basics:

Antibody –

- A **protein** produced by a host in response to the presence of foreign molecules in the body.
- Is synthesized by plasma cells (B lymphocytes) that circulate in the blood and lymph.
- Is a glycoprotein

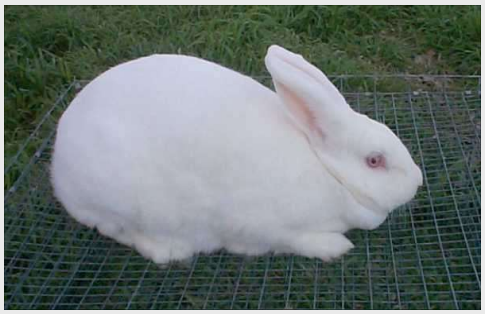
Antigen –

- Any substance capable of eliciting an immune response
  Usually a protein (in our case a viral encoded protein)

Serology: the science dealing with the reactions and characteristics of serum

Basis of serological detection of plant viruses:

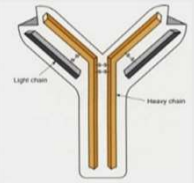
► Antibodies are produced in vertebrate animals or animal cell cultures after they are exposed to plant virus antigens (proteins, virions or virus-specified proteins)



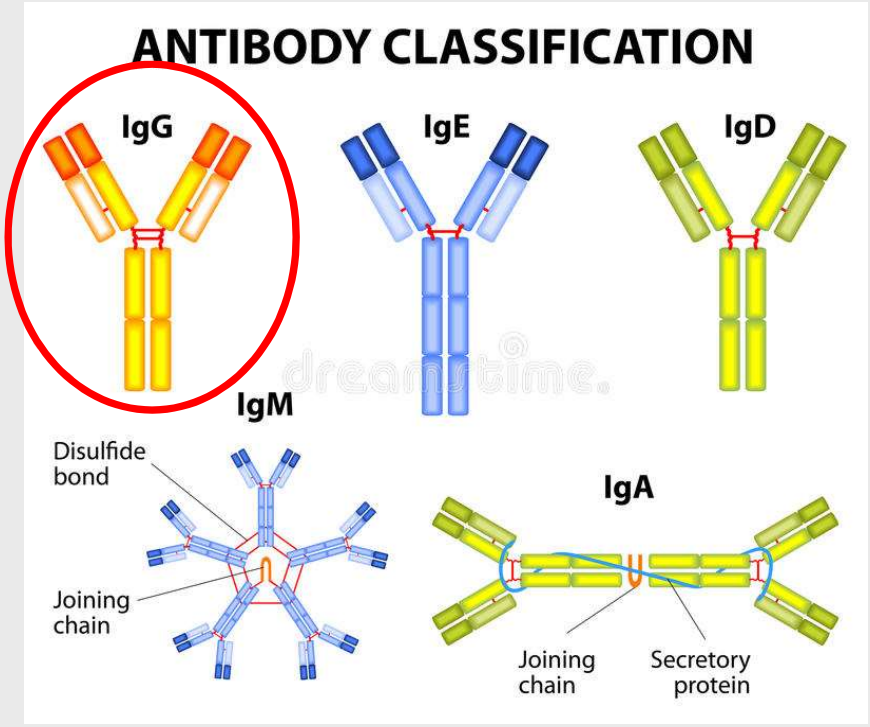
► These antibodies can be extracted from blood or eggs (chickens)

► Antibodies are used in many different assays to detect the proteins (plant viral proteins)

2. Immunology Basics -The Antibody Con't



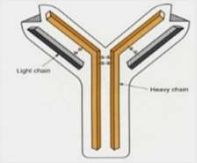
- There are several classes of antibodies which can occur in vertebrates after immunization



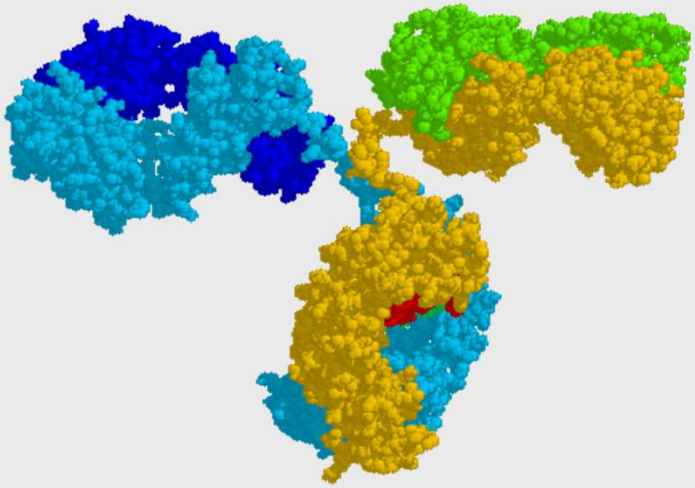
Different types of antibodies have different functions:

IγM primary response
IγG secondary response
IγA protects mucous membranes
IγE protects against parasites
IγY made in egg yolk

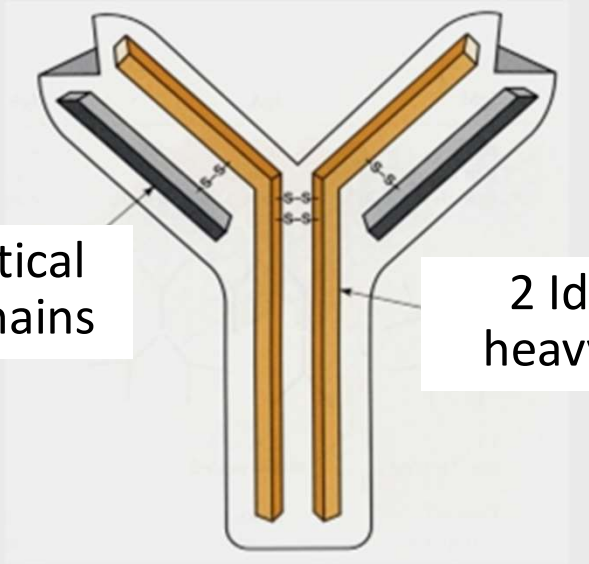
2. Immunology Basics – The Antibody



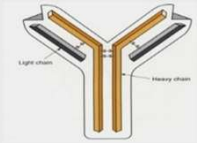
- The most abundant class of antibody in serum is immunoglobulin G (IgG).
- IgG is also the most commonly employed antibody in serological assays.



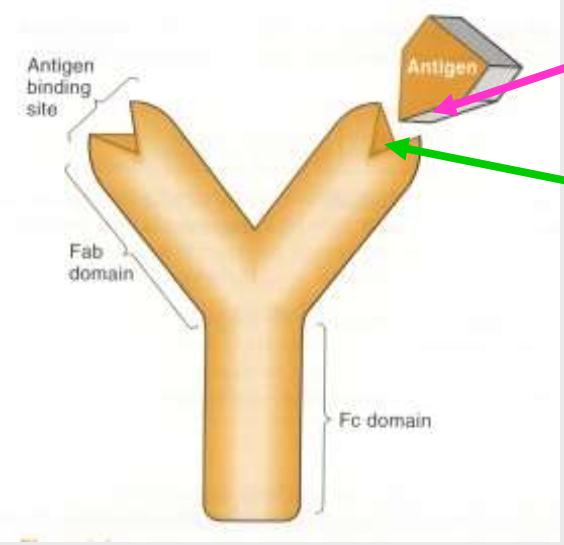
Heavy chains shown in orange and light blue
Light chains shown in dark blue and green



2. Immunology Basics - The Antibody Con't

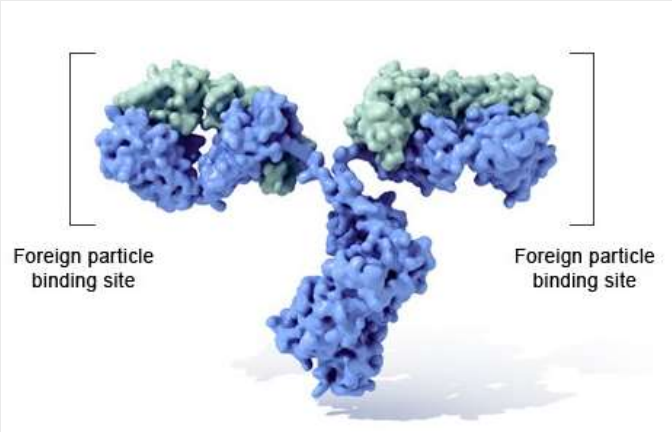


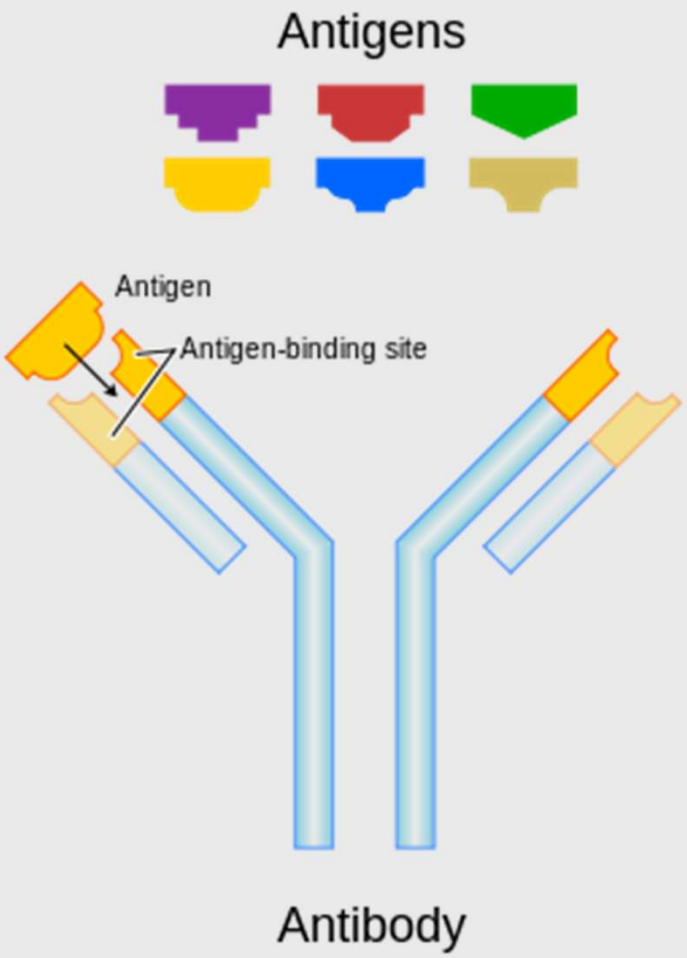
- Antibodies combine specifically with **epitopes** (antigenic determinants) on proteins.



Epitope - the antibody-binding site on the antigen (minimum size ~5-6 amino acids or 6 monosaccharides)

Paratope - the antigen-binding site on the antibody

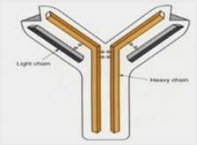


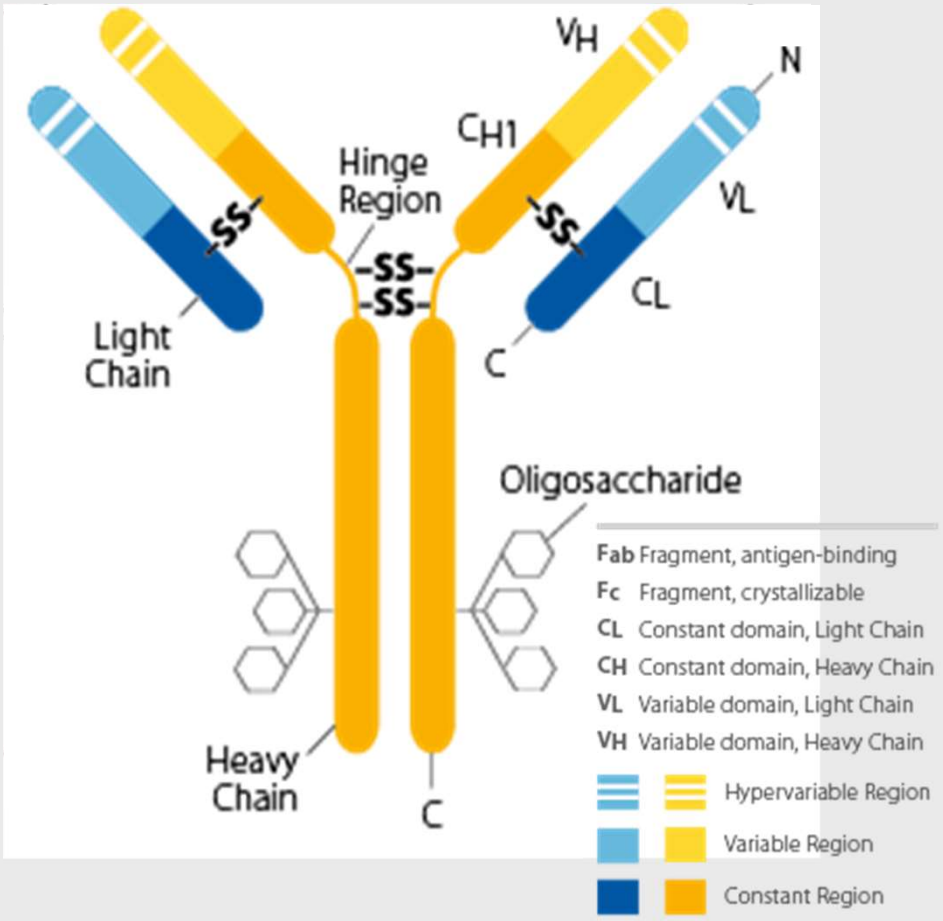


- An epitope binds to the paratope when the 5-6 amino acids of the epitope are recognized by the paratope

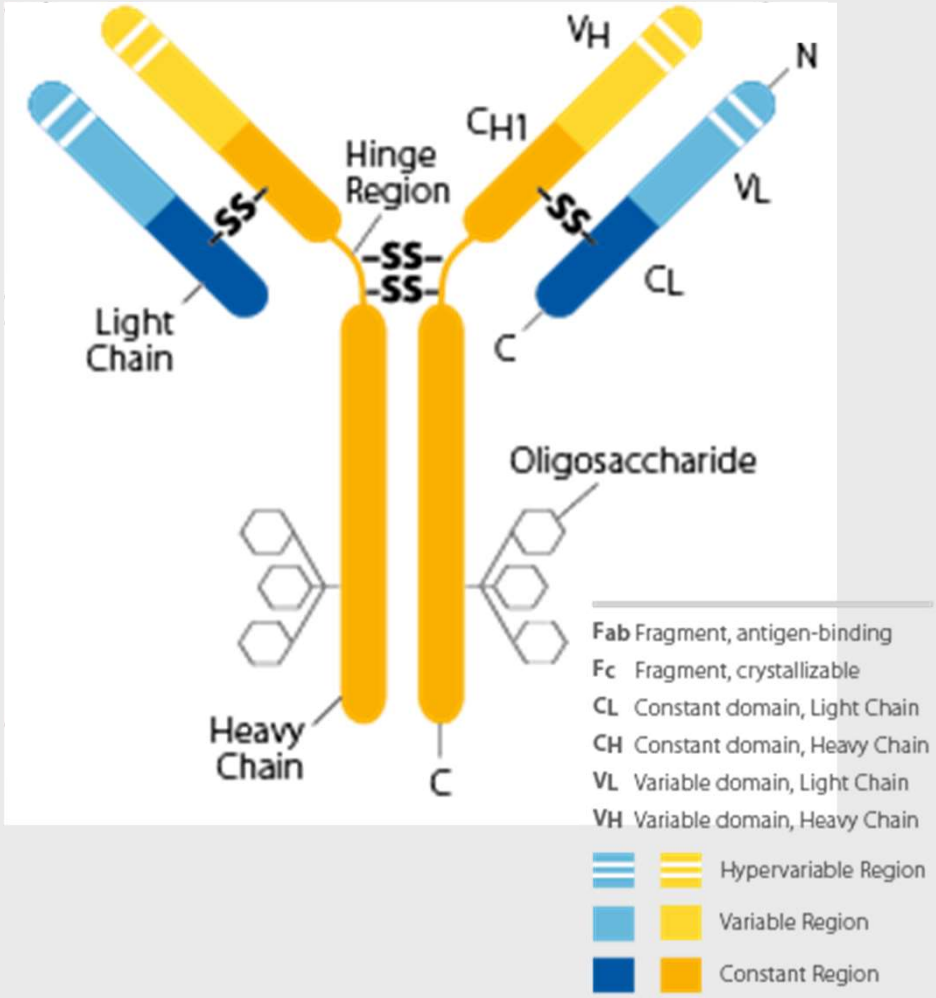
- An epitope binds to a paratope by four types of non-covalent interactions:
  - Ionic or electrostatic forces
  - *Van der Waals forces*
  - Hydrogen bonding
  - Hydrophobic bonding

•2. Immunology Basics - The Antibody Con't





- IgG is a polyprotein with a molecular weight of 150,000 kd
- Heavy chains are twice the size of light chaings
  - light chains are 220 amino acids
  - heavy chains are 440 amino acids
- Light and heavy chains are each produced from genes on different chromosomes

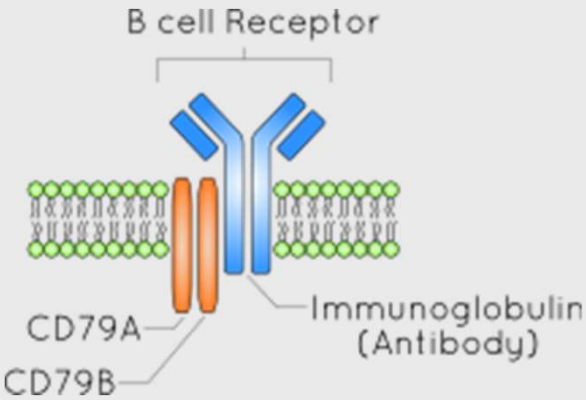


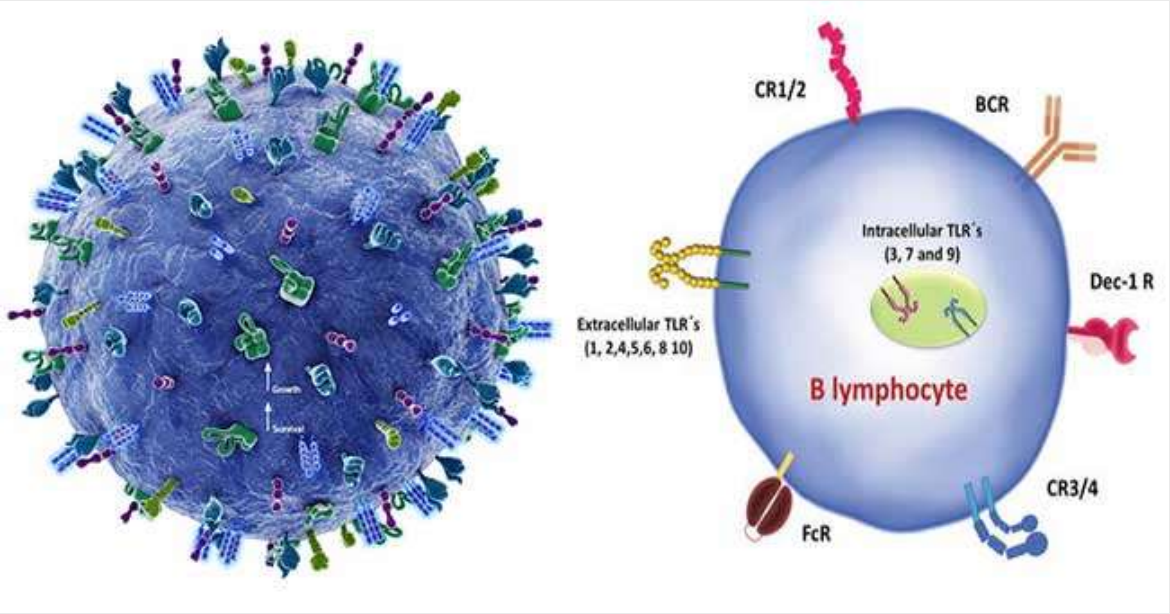
How do antibodies develop specificity to different antigens?

- Antibodies have unlimited potential for diversity.
- Heavy and light chains are produced by different genes
- Roughly half of each chain is encoded by variable genes and the other half by constant genes (located in the chromosomes of B cells).

How do antibodies develop specificity to different antigens?

- Antibodies are secreted by the B lymphocytes (cells of the immune system), each B cell produces one antibody (IgG) against one epitope.

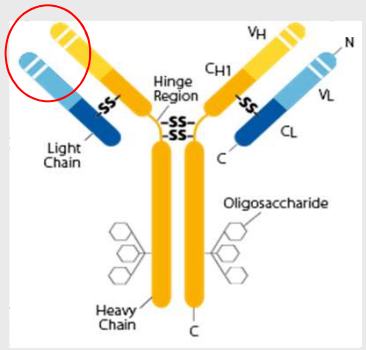




Gearhart, P. J 2002, The roots of antibody diversity, Nature 419, 29-31.

How do antibodies develop specificity to different antigens?

- When a B lymphocyte recognizes and responds to a foreign molecule, the genes that code for the variable regions undergo recombination/mutation at very high frequencies.



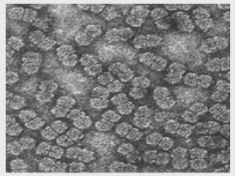
- At first the B lymphocytes produce antibodies of relatively low affinity (not very specific).

- As the immune response progresses, the B cells become hypermutated, resulting in new antibodies some of which can bind the antigen more strongly and specifically.

- Because of the random nature of recombination/mutation different B cells will produce antibodies with different affinities to the antigen

Gearhart, P. J 2002, The roots of antibody diversity, Nature 419, 29-31.

3. Immunology Basics - Antigens



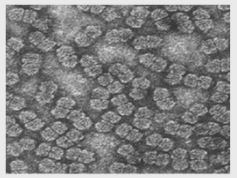
- **Antigens** are usually proteins or polysaccharides;

(lipids and nucleic acids usually do not induce formation of antibodies.)

- **Antigens** capable of stimulating an immune (antibody) response have at least four common characteristics:

(1) **high molecular weight**
(2) **chemical complexity**
(3) **solubility (biodegradability)**
(4) **foreignness**

3. Immunology Basics – Antigens Con't



Sources of Viral Antigens for Immunization:

- **Purified virions:** dissociated (denatured) virions (antigen: coat protein subunits) or native (non-denatured) virions (antigen: intact virion)

- **Purified non-structural proteins**

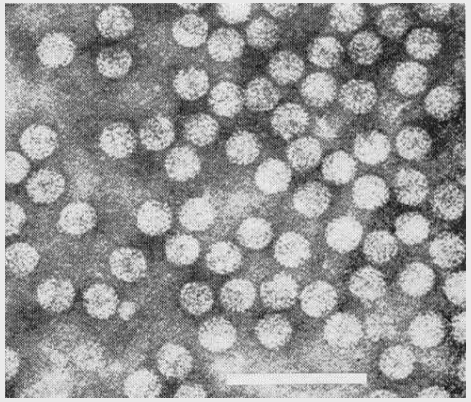
- **Expressed protein:** (structural and nonstructural) from genes cloned and expressed in *E. coli*

- **Nucleic acid vaccines** – a plasmid expressing a viral gene is injected into the host, protein is expressed from the plasmid in the host, and the host produces an immune response. Gets around the need to purify an antigen.

Purification of Plant Viruses:

Goal: Obtain a concentrated, biologically active (infectious) preparation of virions that are free of host components

basically a purification of a complex protein (protein with 3º structure)



Purifying a Plant Virus:

A. Optimize production of virions:

1. Select a purification host
2. Determine optimal timing for inoculation and harvest
3. Have assays to monitor the amount or purity of virus during the process

B. Isolate virions from host:

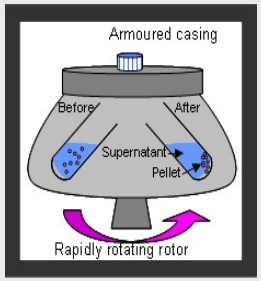
1. Extract

2. Clarify

Remove lipids, pigments, cell walls, cell membranes using cheesecloth and organic solvents

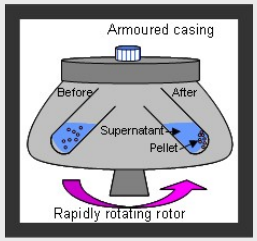
B. Isolate virions from host:

3. Concentrate



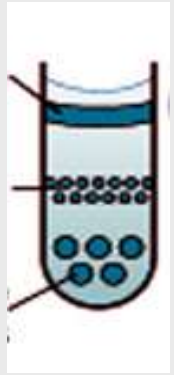
Use a series of gradients and centrifugation to separate virions from plant cell components

4. Final removal of impurities

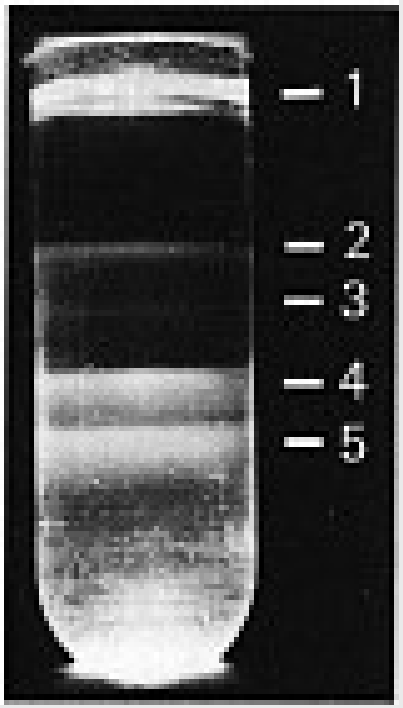


Virions

Cell components smaller than virions



Example: Step 3 (concentration)
Use of density gradient centrifugation to concentrate virions of Cowpea mosaic virus



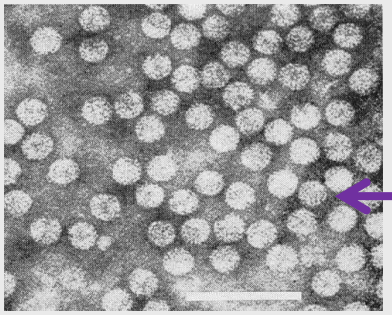
Non-sedimented material (“junk”)

CPMV particles without RNA

Host ribosomes

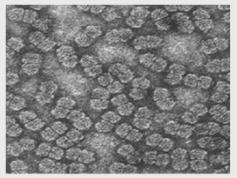
CPMV particles containing shorter genomic RNA

CPMV particles containing longer genomic RNA



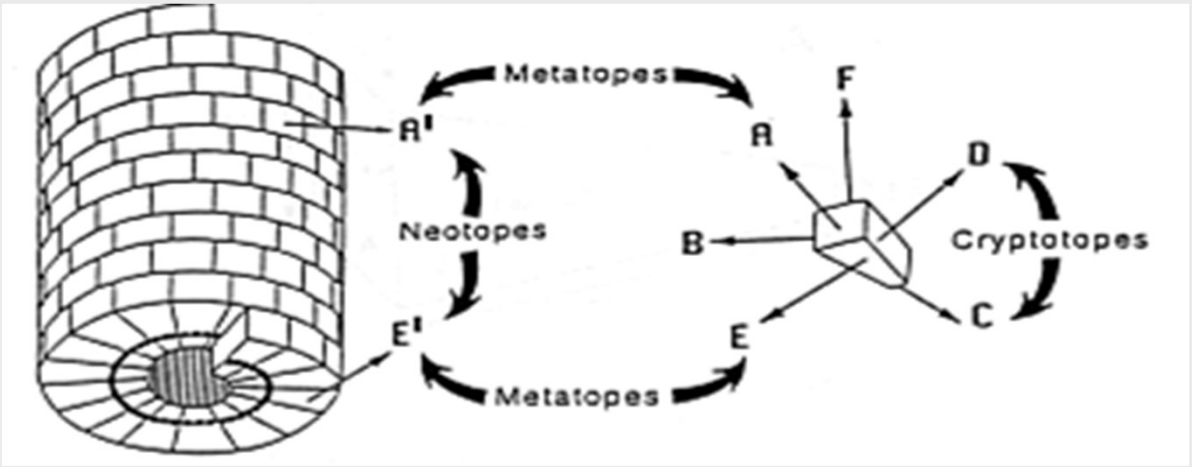
Can be injected into a mammal to produce an antibody

3. Immunology Basics - Antigens Con't



- Intact virus particles or their dissociated capsid protein subunits are the major antigenic targets in serological tests

- Antibodies produced to intact virions and dissociated virions are different.



- Each type of antigen has shared and distinct epitopes so the serum produced by each type of antigen will have some shared and some unique antibodies .

Saunal and van Regenmortel, 1995

HOW DO YOU MAKE ANTIBODIES?

- There are at least 2 different approaches
- Each approach gives you a different type of antibody

Three Basic Types of Antisera:

Polyclonal antibodies (pAbs) – collected from animal inoculated with an antigen with many antigenic sites.

- ♦ consists of a population of antibodies reactive with more than one epitope (prod. by a population of B lymphocytes)
- ♦ requires use of animal immune system
- ♦ collected from the serum of vertebrate blood

Monoclonal antibodies (mAbs) - collected from a single antibody-producing B lymphocyte

- ♦ consists of a single type of antibody which reacts with a single epitope
- ◆ requires use of mouse spleen cells
- ♦ collected from fluid in tissue culture of hybridoma cells or from mouse tumors (ascites fluid)

Polyclonal Antisera

Selection of Host for Immunization

Before injection, viral proteins are emulsified with an **adjuvant,** which helps to boost the immune response, prior to intramuscular injection.

Hosts used for antibody production include:

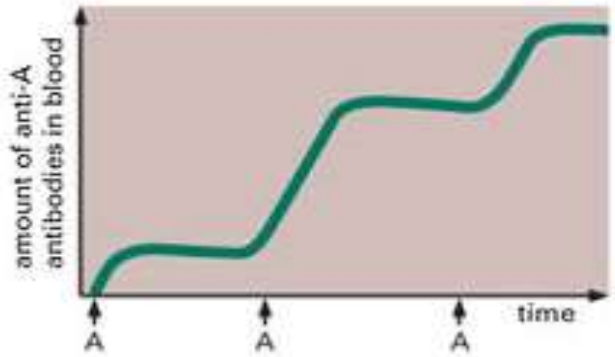
- rabbits,
- rats,
- mice,
- sheep,
- goats,
- horses,
- chickens (egg yolk)

Polyclonal Antisera

inject antigen A

take blood later

Repeated injections of the same antigen at intervals of several weeks stimulates specific B cells to secrete large amounts of anti-A antibodies into the bloodstream.



Because many different B cells are stimulated by antigen A, the blood will contain a variety of anti-A antibodies, each of which binds A in a slightly different way.

Monoclonal Antibodies

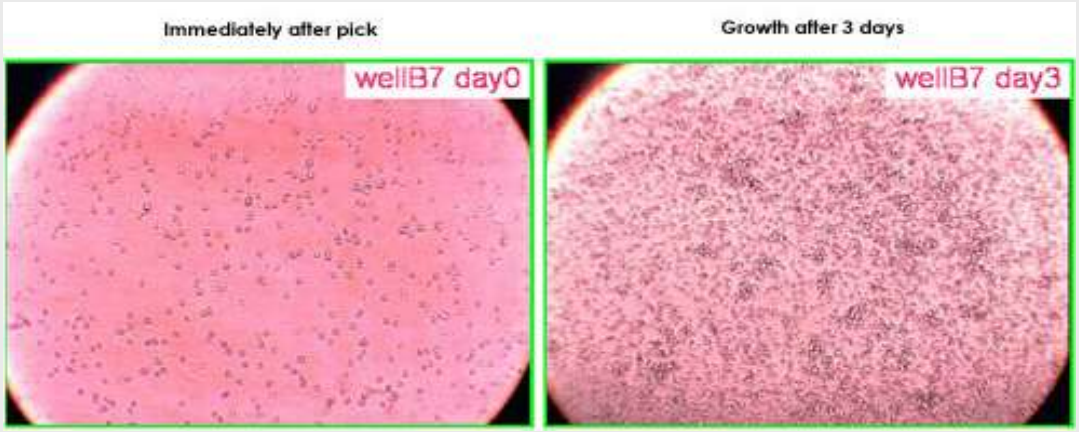
Most monoclonal antibodies are IgG or IgM.
The basic protocol to generate monoclonal antibodies:

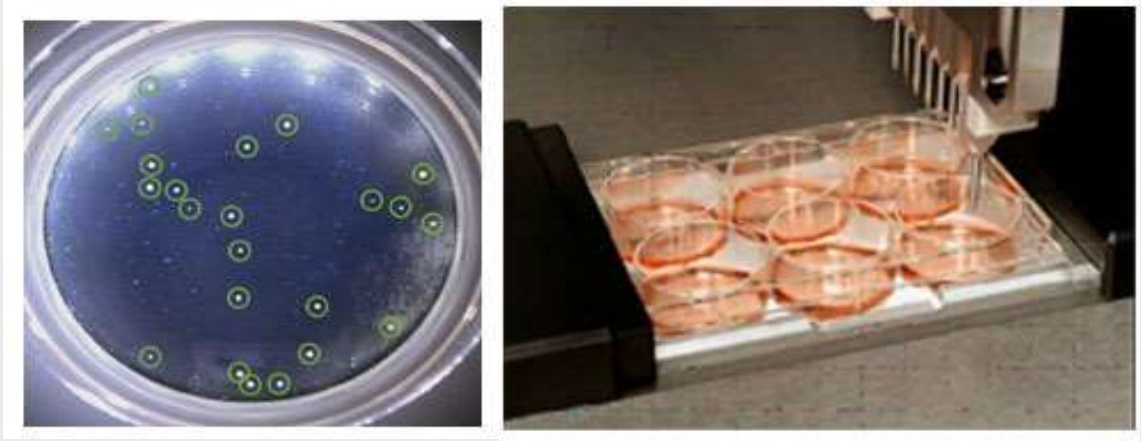
1. **Generate specific immune B-lymphocytes**

   Inject mouse with a series of injections of antigen, verify an immune response, collect mouse spleens and extract antibody-secreting cells (B lymphocytes).

2. **Fuse lymphocytes to a continuously growing myeloma cell line (Hela)**

   this fusion yields a hybrid cell called a **hybridoma**. This fusion immortalizes the lymphocyte. Select individual hybridomas and culture to produce separate cell lines.


Immediately after pick
Growth after 3 days
wellB7 day0
wellB7 day3



Monoclonal Antibodies

3. Identify the desired antibodies in culture supernatants

Screen each cell line for antigen-specific antibody by testing them against the virus antigen of interest.

For the monoclonal antibodies to be reactive in a particular type of assay, it is important to use that assay in the screening and selection process.

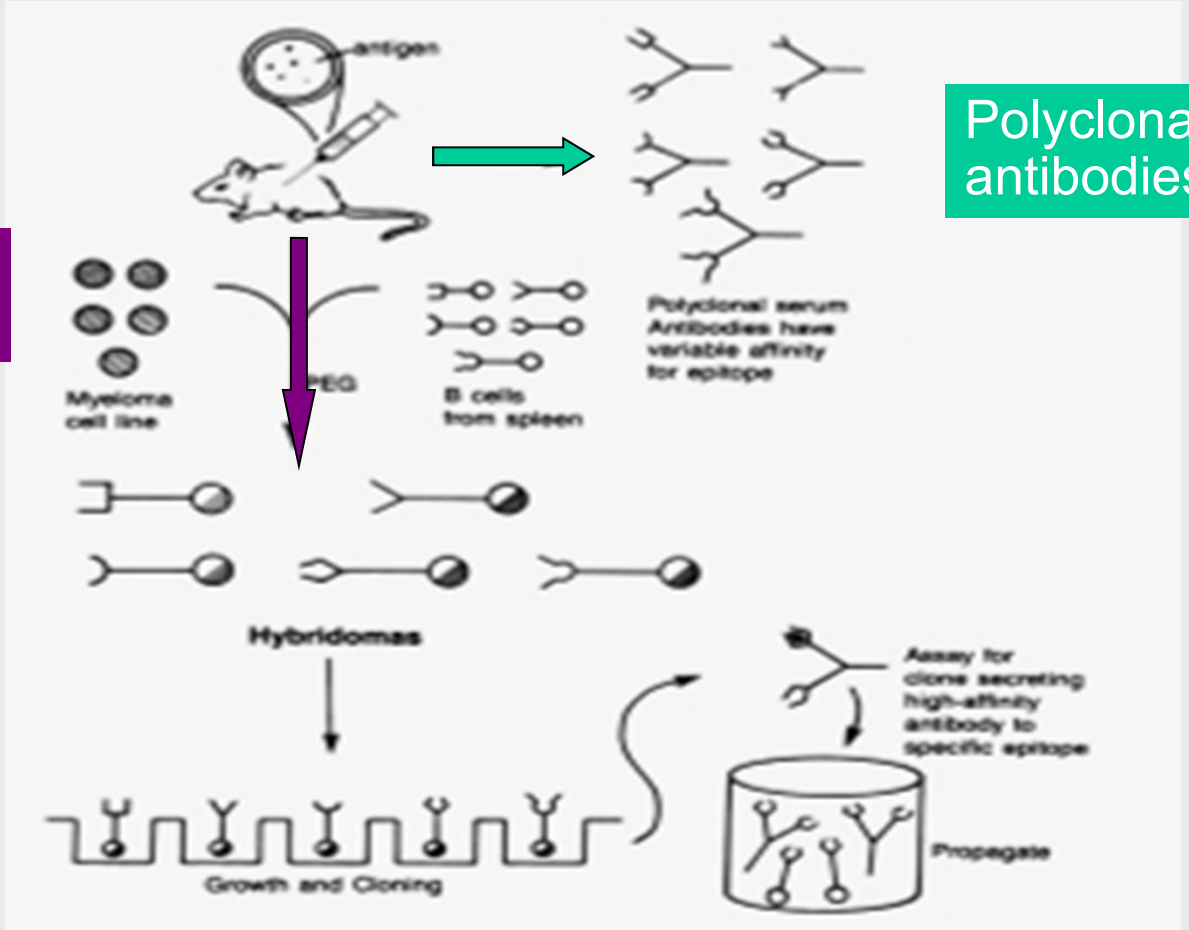
4. Select and culture the selected hybridoma.

5. Extract antibody from hybridoma cell culture and use.

Each hybridoma (and its clones) produce only one type of antibody hence the term monoclonal antibody.

Monoclonal Antibodies vs Polyclonal Antibodies

Monoclonal antibodies



Monoclonal Antibodies

Applications:
monoclonal antibodies (mAbs) can be used in almost any type of assay where you use polyclonal antibodies

mAbs offer several advantages over polyclonal antibodies [pAbs]:

- An **unlimited quantity** and **continuous supply** of antibody can be produced from a small quantity of antigen;

- **Purity** (antibodies specific for a single antigenic determinant can be obtained, even when impure antigen or antigen mixtures are used as the immunogen);

- **Highly specific** can differentiate between plant viruses that were previously indistinguishable using polyclonal Abs);

Monoclonal Antibodies

Disadvantages of mAbs compared with pAbs:

- **Time consuming**
- Production and characterization of mAbs take more than one half year
- **Specificity** of mAbs may limit its diagnostic value
- **More expensive** to produce and require more extensive technology.

Where/How do we make antibodies?

- Make them in-house (at universities)
- Purify or produce the protein and send to companies to make the antibodies

  American Type Culture Collection, Ab-Cam,
  Millipore Sigma (Sigma-Aldrich),
  Europa Bioproducts

- Or:

- Peptides from 2 to 135 amino acids
- Rabbit polyclonal from $1,450 (includes peptide antigen design and 10mg of peptide)
- Mouse monoclonal starts at $11,200

Labeling of Antibodies

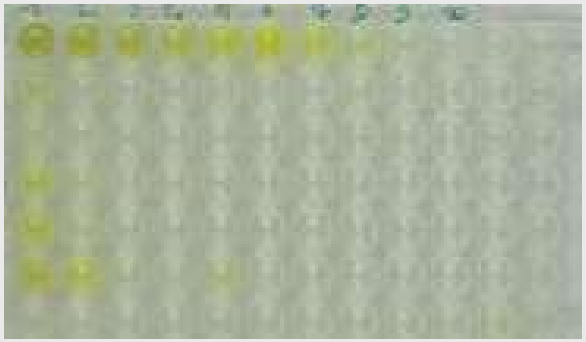
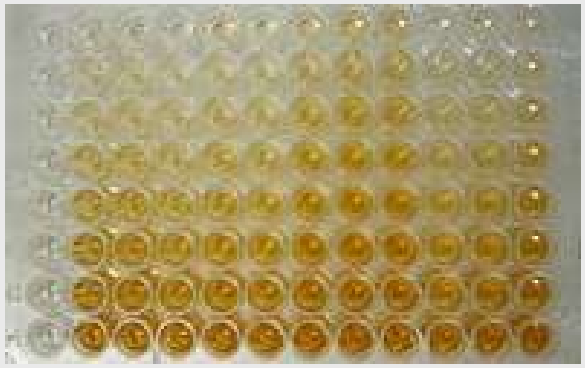
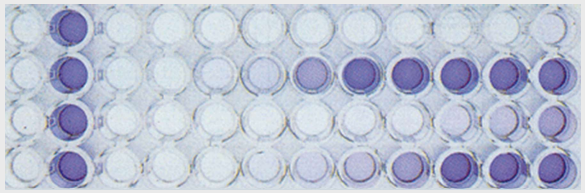
- So your antibody binds to the antigen you are trying to detect….. so how do you know it has bound?
- Your antibody needs to have a “label” (aka a reporter molecule) attached to it and an appropriate means of detecting the reporter molecule
- There are different reporter molecules for different assays and for different purposes

Many Types of Reporters Available For Immunological Assays

LABEL	METHOD OF DETECTION	DISADVANTAGE	ADVANTAGE
Iodine^{125}	X-ray film	Hazardous, Short half-life	Easy to quant., high sensitivity
Enzyme	Chromogenic substrates det. by eye or spectro.	Multiple steps, some hazardous substr., Endo. enzymes interfere	Long shelf life, High sensitivity, Easy to detect
Biotin	Avidin/Strepavidin coupled to various labels	Multiple steps, some hazardous substr., endogenous biotin	Long shelf life, High sensitivity, Easy to detect
Fluorochromes	UV excitation/ emission-Fluor. Micro. or Fluorimeter	Low sensitivity, quenching, auto-fluorescence	Long shelf life, good resolution
Chemi-luminescence	X-ray film	Multiple steps, some hazardous substr.,	Easy to quant., high sensitivity
Biosynthesis	X-ray film	Low sensitivity, Short shelf-life	No damage to antibody

Several Enzymes and Substrates are Commercially Available can be used in many assays

Enzyme (Reporter)	Substrate	Application
Alkaline Phosphatase	Soluble PNPP	ELISA
	Insoluble NBT/BCIP, Fast Red TR/AS-MX	Dot spots, Westerns, Immunocytochem.
Horseradish Peroxidase	OPD, soluble TMB, ABTS,	ELISA
	DAB/Cobalt, Insoluble Chloronaphthol,and others	Westerns, Immunocytochem.
B-Galactosidase	Soluble –ONPG	ELISA
	Insoluble –BCIG, X-Gal	Westerns, Immunocytochem.

	Enzyme	Substrate
	Alkaline phosphatase	PNPP
	Horseradish peroxidase	OPD
	B-Galactosidase	ONPG

Commercial Sources of Antibodies for Plant Viruses:

ADGEN SAC, Diagnostic Systems, Auchincruive, Scotland	www.adgen.co.uk
AGDIA, INC. , Elkhart, IN	www.agdia.com
BIOREBA AG, Reinach, Switzerland	www.bioreba.com
LOEWE BIOCHEMICA GmbH Sauerlach, Germany	www.loewe-info.com