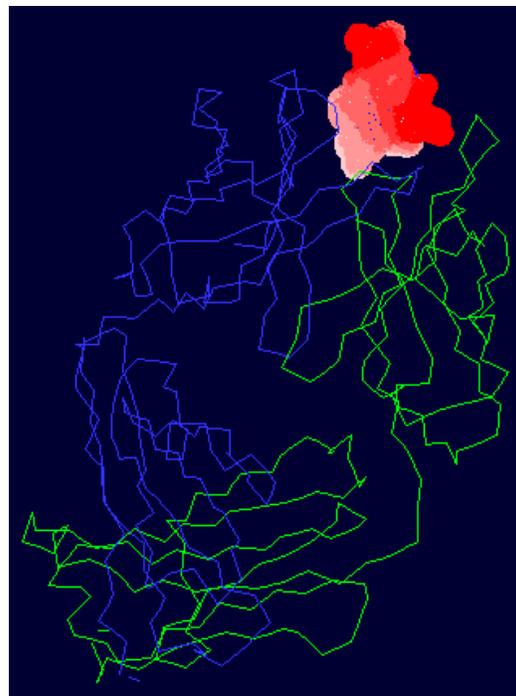


## IMMUNOLOGICAL TECHNIQUES: MOSTLY ANTIBODIES

**POLYCLONAL AND MONOCLONAL.** When you work with antibodies, you will use either polyclonal antisera, or monoclonal antibodies. **Polyclonal** antisera are made in the traditional way, by administering antigen several times to an animal (person?) and then drawing blood, letting it clot, and separating the serum. Adjuvants are usually added to the antigen; these stimulate the innate immune response leading to dendritic cells that are more fully loaded and activated. Complete Freund's Adjuvant, made up of oil, emulsifier, and dead *Mycobacteria*, is nasty<sup>1</sup> but a favorite. You can also immunize hens and recover startling amounts of antibody—it is called IgY—from the yolks of their eggs!<sup>2</sup> (Several companies offer to do this for you.) The good things about polyclonals are: You can easily make them yourself without special equipment; many scientists have even immunized their own pets, cows, horses, or selves. You can boost until the titer is very high, and by bleeding early instead of late you can often get more IgM and less IgG if that's your preference. You will control how specific the antiserum is by the purity of your injected antigen preparation. But remember the amplifying ability of the immune response; you may get a lot of antibody to a minor contaminant. If so, and you have some precious ultrapure antigen, you can couple it to a solid support such as Sepharose<sup>®</sup> and use it to absorb out the antibody you want, washing away all other specificities, and then releasing your antibody from the immunosorbent with acid or high salt. This is referred to as **affinity-purified antibody**. A preparation like this is wonderful, as it can be quite specific, and react strongly with the antigen because it consists of the products of several B cell clones, some reacting with different epitopes, or differently with the same epitope. They are superior, for example, in immunohistochemistry and immunoblotting, where some of the epitopes may have been damaged by fixation or denaturation. The problem with polyclonals is that once you use up a batch, it's gone; and no one else, including you, can make exactly the same serum again. So for critical procedures, monoclonals from a single clone source are often a better choice.

**EPITOPOLOGY.** The **epitopes** that bind to the antibody's combining sites are typically made up of 10 to 20 amino acids. They are of two types: The more obvious **linear** or **continuous epitopes** are a single stretch of a protein sequence, for example this peptide, KPLEEVLNL<sup>3</sup>, which is a continuous segment (64-72) from human interleukin 2. It is shown here in red (the blob at top right), being bound by V<sub>L</sub> and V<sub>H</sub> antibody domains.

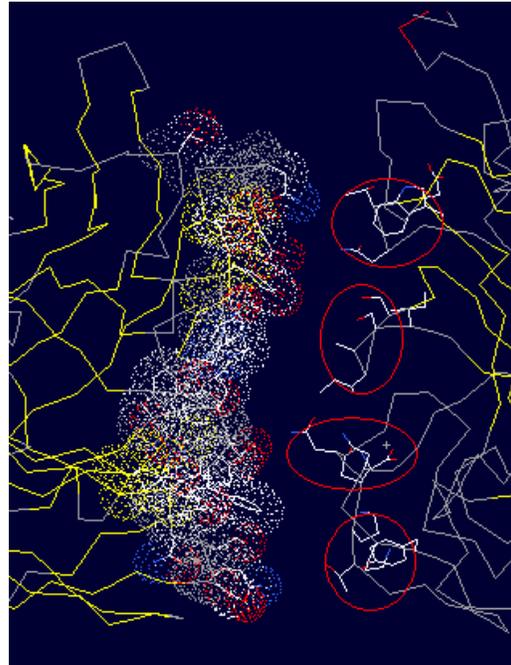


<sup>1</sup> <http://research.uiowa.edu/animal/?get=adjuvant>

<sup>2</sup> [http://www.gallusimmunotech.com/What\\_is\\_IgY](http://www.gallusimmunotech.com/What_is_IgY)

<sup>3</sup> See page 6 for single- and three-letter abbreviations.

More common are **conformational** or **discontinuous epitopes**. These consist of amino acids that are close together in the tertiary structure of the native, folded protein, but are not contiguous in the primary sequence, as in this epitope from influenza virus neuraminidase. On the left is the antibody combining site, with the amino acids of the complementarity-determining regions in ghostly van der Waals depiction. On the right, pulled away a few angstroms for clarity is the epitope, with its 4 discontinuous contact zones PNDPT(328-332) + YPGN(341-344) + ISIAS(366-370) + NTDW(400-403) circled in red.



Pictures from the Kyoto Continuous Epitope Database (no longer on line); a good web resource is at <http://www.immuneepitope.org>

You need to keep in mind that, if your antibody was made against a native protein, you can't necessarily expect it to react with a protein that you've boiled in detergent, electrophoresed and electroblotted, and baked in an oven. Maybe you will get lucky, but this is where you have to read labels carefully and learn what others have already found out about that serum or monoclonal.

**MONOCLONAL ANTIBODIES (mAbs).** The history of this development is interesting and you can read about it elsewhere<sup>4</sup>. You've heard a lot about these already; let's take a moment to discuss them some more. The idea that occurred to Milstein and Kohler was: wouldn't it be nice to have a cell line that made antibody like a B cell, but lived forever like a tumor? Here's how it's done, using newer tools that weren't available then. You immunize a mouse with some antigen, take out the spleen at the height of the B cell response, and tease it apart in cell culture. Then you take a mouse multiple myeloma—malignant plasma cell—line which has mutations induced in it so that, while it still grows like a tumor that knows how to make a lot of antibody, it has lost its structural genes for H and L chains; furthermore, it has an enzyme defect so that it can't survive in a particular growth medium which is adequate for normal lymphocytes. Mix the spleen cells and the myeloma, and add an agent which causes cell fusion: polyethylene glycol. The cells are then left in culture in the special medium for several

<sup>4</sup> Rajewsky, Klaus. *Nature* **416**, 806 (25 April 2002) Obituary: César Milstein (1927–2002). See Supplementary Material on the course website. When Milstein told him in 1975 about monoclonals, Rajewsky remembers, "I felt the earth shaking." Kohler and Milstein shared the Nobel Prize in 1984.

days. Consider: B cells which have not fused with anything, or which have fused with other normal cells, will die, because normal cells are mortal, especially B lymphocytes in tissue culture. Unfused myeloma cells similarly will die in the inadequate medium. *Only fusions* between normal cells (which provide the enzyme needed to survive) and myelomas (which provide immortality) will survive and grow<sup>5</sup>. We don't care if some of the fusions involve non-B cells. We dollop a few cells out into each of many tiny culture wells, and after a few days check the supernatant fluid for antibody. If we've been lucky, there will be some wells making exactly the antibody we want. Those wells contain at least one hybrid, and we can go on to clone it out. It can then be transplanted into mice of the appropriate strain, or carried in culture, and in bulk produce grams, even kilograms, of antibody. The final producing cell is called a B-cell **hybridoma**.

These antibodies derive from the progeny of a single B cell so they are truly monoclonal. Think what you could do with an unlimited quantity of antibody to any particular determinant, of utter specificity and any desired affinity. Thousands are used in labs around the planet, and 34 are already FDA-approved drugs or in-vivo diagnostics.

**MURINE, CHIMERIC, HUMANIZED, HUMAN MONOCLONALS**<sup>6</sup>. Most mAbs are made by immunizing mice, which are cheap, good responders, sufficiently distant from humans (75 million years) to make good anti-human responses, and have 50-100 million B cells per spleen. Many mouse mAbs react with mouse proteins! This is probably fairly low affinity cross-reaction; it wouldn't be noticed in a polyclonal response, but since the mAb production process samples every B cell in the spleen, even rare cells can get selected<sup>7</sup>. Hamster mAbs can be made against mouse antigens if necessary. Rat mAbs have been made, though the rat B cell is reluctant to fuse. A new company, Epitomics, specializes in rabbit monoclonals, which it claims, probably correctly, have higher specificity and a wider repertoire (bigger immune system) than those of the mouse.

Muromonab-CD3, the first mAb to be used in humans, is against CD3, a pan-T cell marker, and is used to temporarily eliminate T cells from transplant recipients. But it is **murine**<sup>8</sup> IgG, and thus it was found to be difficult to use it twice; patients make their own antibodies against it. These are called HAMA, human-anti-mouse antibodies. So the jocks took their mouse hybridomas, isolated the H and L chain genes, and recombined the gene segments for the V<sub>H</sub> and V<sub>L</sub> domains of the antibody with the C<sub>L</sub> and C<sub>H</sub> regions of a human IgG gene. The recombined genes were inserted into an appropriate cell line, which produces a **chimeric** antibody: with mouse V<sub>H</sub> and V<sub>L</sub> and everything else human, it would be only about 33% mouse. Such antibodies can be used more than once, but not too often, because HACA develop. So the jocks went back and cut just the sequences for the CDRs out of the mouse mAb genes, and inserted them into the framework of human IgG V<sub>H</sub> and V<sub>L</sub> domains, then appended all the human C domains, to make a **humanized** mAb; some of these have as little as 2% mouse sequence, and though HAHA have been

<sup>5</sup> Specifics in **Supplementary Material: Making a hybridoma**.

<sup>6</sup> See **Supplementary Material: How to name a monoclonal antibody**.

<sup>7</sup> Unlike PowerBall, where your chances to win are slightly higher if you don't actually buy a ticket.

<sup>8</sup> *Mus, muris* Latin, murids (rats & mice.) Immunologists only care deeply about mice, so for us murine = mouse, usually.

described, the antibodies are usually able to be used repeatedly. Finally, fully **human** mAbs arrived a few short years ago. They can be made in humanized mice (immunodeficient mice reconstituted with human hematopoietic stem cells, fetal thymus, and lymph node) or you can immunize a human and try to immortalize his or her blood B cells with Epstein-Barr virus<sup>9</sup>.

That isn't the end. Lately several companies, especially MedImmune, have tinkered with certain amino acids in the Fc end of their hybridomas, greatly changing the ability of the antibody to be bound by different Fc receptors. This affects not only binding to phagocytes, ADCC<sup>10</sup>, etc., but also the half-life of the monoclonal Ab, which may be significantly prolonged, increasing efficacy.

Or you can get rid of Fc entirely. Many groups have made single-chain antibodies by cloning the VH domain, a short linker, and then the VL domain, from a single antibody. The linker allows the two variable domains to align and the specificity is retained. A few of these have seen clinical trials. They're referred to as **scFv**, single-chain variable fragments.

## A COUPLE OF ILLUSTRATIVE TECHNIQUES:

### 1. IMMUNOPRECIPITATION.

We've already discussed how antigen-antibody complexes grow and, at optimal proportions, precipitate. A modified technique is very common in research labs, where it is used to isolate proteins of interest and sometimes see what other proteins might be associated with them (that latter is called coimmunoprecipitation). Let's imagine the following experimental question: When mice are forced to watch *CSI: Miami* on TV, does their brain concentration of the transcription factor Borin increase? And does Borin associate with the sleep-inducing cofactor Yawn? We expose mice to nothing or CSI:M, and make brain extracts. To each we add rabbit antibody to mouse Borin. We incubate with stirring for a while, and then add Sepharose<sup>®</sup> beads to which Staphylococcal Protein A or Streptococcal Protein G has been bound. These proteins happen to have high affinity for the Fc portion of IgG. So all antibodies in the solutions are bound by the beads, carrying along any antigens that might be bound to *them*. You spin out the beads, wash them, make them up in loading buffer, and run them on polyacrylamide gels. And what do you see? From the control extract, a faint band at 61 kDa, the molecular weight of Borin<sup>11</sup>; in the CSI:M condition, a strong Borin band (immunoprecipitated), and another band at 19 kDa, the size of Yawn (coimmunoprecipitated.) *Nature Weird Neuroscience*, hold the press!

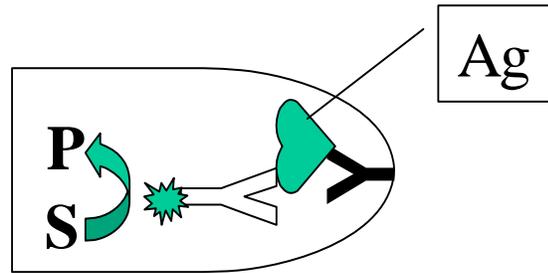
<sup>9</sup> See, if you have time or are interested, **Supplementary material: How to make human monoclonals.**

<sup>10</sup> Antibody-dependent cell-mediated cytotoxicity, discussed in the Tumor Immunology unit.

<sup>11</sup> You often see 25 kDa light chain and 50 kDa heavy chain bands from your antibody, too.

## 2. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

If an antigen is at least *divalent*, a favorite technique to measure its concentration in a biological fluid or other solution is a **sandwich or capture ELISA**. Let's say it's a patient's serum and we want to measure myocardial creatine kinase isoform MB in the serum, that might be elevated shortly after a heart attack. We start by making or buying two monoclonal antibodies to human CK-MB, each to a different epitope. Put one mAb in the bottom of a well in a multiwell plate so that it's stuck there. Though they stick



head-up and head-down, we assume that at least some of the antigen-binding sites are available. Add the patient's serum. Wash off anything that isn't bound. Then add the second antibody, which will stick to the other epitope on the antigen in proportion to how much antigen's there. The second antibody has an enzyme  coupled to it—usually peroxidase—and it completes the sandwich. Now add a colorless peroxidase substrate (**S**) that produces a colored product (**P**). Finally, measure the intensity of the product color in a plate spectrophotometer, also called an ELISA reader.

We use the enzyme-labeling technique because it's easy to detect and doesn't involve radioactivity. There are variants which use a substrate that becomes a fluorescent product; they're more sensitive, but the equipment is more expensive.

Please note that there are other kinds of enzyme immunoassay. For example, the common ELISA screening test for AIDS measures antibody to HIV, not an antigen. In this case, crude HIV antigen (extracts of infected T cells) is coated on the bottom of the plate. Patient serum is added, and unbound material washed away. Any IgG that adhered to the antigen is detected with an enzyme-bound animal antibody to human IgG.

Enzyme immunoassays, with their simple repetitive pipetting-washing steps, lend themselves to high-throughput robotic testing.

## Amino Acid Abbreviations

### Nonpolar (hydrophobic)

| amino acid    | three letter code | single letter code |
|---------------|-------------------|--------------------|
| glycine       | Gly               | G                  |
| alanine       | Ala               | A                  |
| valine        | Val               | V                  |
| leucine       | Leu               | L                  |
| isoleucine    | Ile               | I                  |
| methionine    | Met               | M                  |
| phenylalanine | Phe               | F                  |
| tryptophan    | Trp               | W                  |
| proline       | Pro               | P                  |

### Polar (hydrophilic, H-bond forming)

|            |     |   |
|------------|-----|---|
| serine     | Ser | S |
| threonine  | Thr | T |
| cysteine   | Cys | C |
| tyrosine   | Tyr | Y |
| asparagine | Asn | N |
| glutamine  | Gln | Q |

### Charged (negative, hydrophilic)

|               |     |   |
|---------------|-----|---|
| aspartic acid | Asp | D |
| glutamic acid | Glu | E |

### Charged (positive, hydrophilic)

|           |     |   |
|-----------|-----|---|
| lysine    | Lys | K |
| arginine  | Arg | R |
| histidine | His | H |

## **Learning Objectives for Immunological Techniques**

1. Define epitope, and describe the difference between continuous and discontinuous epitopes. Discuss practical consequences of these differences.
2. Define adjuvants, and describe their function.
3. Define monoclonal antibodies, and describe in principle how they are made.
4. Compare and contrast murine, chimeric, humanized, and human monoclonal antibodies. Discuss which might have disadvantages when used in human patients, and the reason for that.
5. Define immunoprecipitation and coimmunoprecipitation, and describe how the techniques are performed, and when they might be useful.
6. Describe the technique and use of the ELISA.