IMMUNOCHEMICAL TECHNIQUES

Disclaimer: These handouts are revised to some degree each year. Even so, it is not possible for me to keep on top of every new technical development and product. Nor is it necessary. I am giving you a good basis for understanding and evaluating methods, reagents and instrumentation which happens to be available if and when you need to use methods of this kind. It will also help you evaluate the data from such methods presented in scientific reports. Any specific companies and products which are mentioned are given as examples. They may not be the best products available for your purposes. Companies and products change. Research it for yourself.

ANTIBODIES

In class we discussed what antibodies were, their modular multi-functional structure, how the great diversity of antigen binding surfaces is generated by immunoglobulin gene rearrangement (heavy and light chain V (D) J segments) in B lymphocytes, and how different isotypes are produced by switching the use of heavy chain constant region gene segments. We looked at some of the different properties associated with different isotypes, and noted that, except for a limited range of special T-independent antigens, antibody production requires helper T cell responses. In contrast to the B cell antigen receptor (surface Ig), the T cell antigen receptor recognizes only some small fragment of foreign protein (peptide) presented to it in association with a major histocompatibility complex (MHC) protein by antigen-presenting cells (APC). To be helped by an activated helper T cell, a B cell must present the same peptide-MHC protein complex, so the foreign protein must be associated with the B cell antigen during immunization. This is why small B cell antigens (e.g. organic chemicals, oligopeptides), called haptens, are usually coupled to protein carriers for immunization. Some oligopeptides may also be good T cell antigens, but the other function of the carrier is still important: concentrating the hapten on larger molecules which are not so readily cleared from the body, and which are more efficiently taken up by antigen presenting cells.

IMMUNIZATION

Immunization procedures have developed primarily because they work. Vaccination is an important area of medical research, and some of the results may be useful for those raising antibodies for research, but the goals are different. One is for effective and long term immunity, while the other is for short term high titre and high specificity of antibody.

Normal antibody levels in the blood are maintained between 1 & 5mg/ml.

Because the potential repertoire of B cell (antibody) and T cell specificities is restricted in an individual by mechanisms which guard against production of autoimmune effector cells, there are occasions, especially with simple antigens and immunization of inbred animals, when one individual will generate a response but another will not. A different carrier may overcome this effect for antibody production. If the antigen is very similar to a naturally occurring (self) molecule in the animal to be immunized, one may need to use a different animal species.

Antigen may be introduced through various routes, and the route chosen can affect the type of antibody response, and the optimal amount of antigen required. Typical routes are by injection i.v., i.p., i.m., i.d., or s.c. (intravenous, intraperitoneal, intramuscular, intradermal, subcutaneous). Antigen introduced through the gut is more likely to elicit an IgA response. The response to SRBC (Sheep red blood cells), for example, requires 1/10th as much antigen i.v. as i.p.. In the case of i.m., i.d., s.c. immunizations, antigen is best introduced at several sites.

Newborn animals do not mount strong immune responses and are easily made tolerant to antigen.
High doses of antigen may be tolerogenic, especially if levels are maintained in the recipient for several days.

An antigen preparation containing low levels of contaminants may still produce a highly specific response if injected in small amounts. But, if large amounts are injected, the response to the major component may be compromised, and responses to contaminants may even exceed that to the intended antigen.

High levels of antibody against the immunizing antigen will facilitate its clearance and inhibit the immune response. It is advisable to wait until a prior response has subsided before injecting a boosting dose of antigen. A primary response is relatively weak (and IgM), but the animal should be rested (typically 8 weeks, although 4 week intervals are often used) between boosts.

Complex antigens like whole cells are usually very immunogenic, but good responses to weakly immunogenic molecules may be obtained by injecting with adjuvant.

Adjuvants reduce the solubility of the antigen so it is not so quickly cleared, and, ideally should act upon the immune system to potentiate specific responses and tip the balance against tolerance induction. More likely they induce a background polyclonal response which provides a lymphokine environment beneficial to the progress of the antigen specific responses.

Note: the highly effective and commonly used Freund's complete adjuvant, which contains killed mycobacteria, should be used only once, with the primary injection, otherwise anaphylactic shock may result. Subsequent injections may be in Freund's incomplete adjuvant (lacking the mycobacteria). Furthermore, be very careful not to stab yourself with a needle containing any Freund's adjuvant! (If you do, squeeze and wash immediately, and seek medical advice). Other safer adjuvants are available, and should be the first choice unless good reason is found to indicate that Freund's is needed. Such adjuvants are aluminium salts, Ribi system of monophosphoryl lipid A and trehalose dimicolate (available from Cedarlane), and Hunter's TiterMax (from CytRx corp., Georgia). Liposomes have been used (Gregoriadis G. 1990 Immunology Today 11:89-97). Watch for new developments. Animal Care personnel encourage you to consult with them on such matters, and they have standard procedures for antigen volumes, routes of administration, bleeding techniques and blood volumes for each of the common species used for antibody production.

Small simple molecules (hormones, peptides, chemical haptens) may be rendered immunogenic by coupling them to larger, carrier, molecules. Such coupling is usually considered necessary for polypeptides of MW<3,000, and is probably beneficial for any with MW<10,000.

Synthetic oligopeptides can be synthesized using the MAP system (multiple antigen peptide), which results in several synthetic peptides supported on a lysine backbone. In this case, a carrier may not be needed. Peptides, both single, and in a MAP array, can be synthesized at the McGill Sheldon Biotechnology Centre. (The Centre will even arrange to produce rabbit antisera for you). If you are having oligopeptides synthesised in the hope of raising antibody that will detect a large protein which contains that amino acid sequence, be aware that the anti-peptide antibody may not bind the native protein, but will likely bind the denatured protein.
**HAPten-carrier Coupling reactions.**

Haptens, e.g. Hormones, Drugs, Carbohydrates, are coupled to carriers to reduce the rate of clearance, and usually to provide sufficient associated protein for an efficient helper T cell response. Here are some frequently used carriers (Table on next page):

<table>
<thead>
<tr>
<th>Carrier</th>
<th>OD(280), 1mg/ml</th>
<th>M W (approx.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (Bovine serum alb.)</td>
<td>0.68</td>
<td>66,000</td>
</tr>
<tr>
<td>cBSA (cationized BSA)</td>
<td>(Pierce Chem Co.)</td>
<td>(&quot;Super carrier&quot;)</td>
</tr>
<tr>
<td>BGG, CGG, or HGG</td>
<td>1.4</td>
<td>150,000</td>
</tr>
<tr>
<td>(Bovine, Chicken or Human gamma globulin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KPH (Keyhole limpet haemocyanin)</td>
<td>2.0</td>
<td>8-9 million at pH &gt;7</td>
</tr>
<tr>
<td>Ficoll, AECM-Ficoll</td>
<td>Low</td>
<td>400,000</td>
</tr>
<tr>
<td>(Aminoethyl-carboxymethyl-Ficoll, Biosearch)</td>
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<td></td>
</tr>
<tr>
<td><em>No protein component.</em></td>
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</table>

Must assay Ab to hapten on different carrier because of anti-carrier antibodies present. May need to absorb out the anti-carrier activity. To be sure free hapten is detected, do a competition assay.

Often a drug analogue is specially synthesized with a reactive group on it through which it may be coupled to the carrier. Carboxyl groups may be reacted with N,N-dicyclohexylcarbodiimide and N-hydroxysuccinimide to produce N-hydroxysuccinimide esters which react with amino groups.

The reagent SPDP (N-succinimidyl-3-(2-pyridylthio)propionate) reacts with amino groups to introduce 2-pyridylthio groups onto hapten and/or carrier. These react with thiols to form disulfide cross-links. If both hapten and carrier are reacted with SPDP, then the 2-pyridylthio groups on one of them may be reduced to thiol groups prior to mixing with the other. This coupling scheme is also useful for conjugating antibodies with enzymes or other proteins useful in immunoassays.

Carbohydrates can be activated by reaction with cyanogen bromide. They then couple spontaneously to amino groups.

Many other coupling reactions are available. Scan the literature when you have a need to know. Also contact Pierce Chemical Co. for its latest edition of Double Agents bulletin.

These coupling reactions are also used to covalently link tag molecules such as biotin, enzyme, or fluorescent groups to antibodies, and to couple antibodies to activated solid support such as dextran, agarose, coated glass beads, or coated paramagnetic beads for example, for use as affinity matrices in immunoaffinity chromatography of molecules or cell fractionation.

When native proteins are used in the immunization, one should expect antibodies which recognize epitopes on the surface of the native molecule. There may also be a useful amount, or practically no antibody able to bind to the denatured protein (e.g. on Western Blots of SDS (sodium dodecyl sulphate) denatured 2-ME (mercaptoethanol) reduced proteins).

In general, consider what you want the antibody preparation to do for you, and select the immunogen accordingly.
MONOCLONAL ANTIBODIES AND HYBRIDOMAS

Monoclonal Antibodies

The following are some notes taken from "Monoclonal Antibodies: Principles and Practice", James W. Goding, Academic Press, 1983.

Immunization with Protein Antigens:

First injection should be in highly aggregated form. Subsequent injections may be either soluble or aggregated.

Adjuvant is usually necessary with water soluble protein. Freund's complete adjuvant is most used. Antigen may be precipitated on aluim with or without the addition of killed Bordetella pertussis. Use of adjuvant may influence class of antibody. Handman and Remington (1980) Immunology 40: 579- 588; hybridomas from spleen cells of mice chronically infected with Toxoplasma gondii are mostly IgG2&3 secreters, reflecting the serum class distribution of specific antibodies, whereas Johnson et al (1981) Aust. J. Exp. Biol. Med. Sci. 59: 303-306, got many IgG1 hybridomas after injecting T. gondii in Freund's. If the antigen is of very low molecular weight, it may be made more immunogenic by polymerizing it or coupling it to a carrier molecule.

A reasonable schedule would be 10 micrograms emulsified in 0.2 ml complete Freund's i.p., boosted twice with same dose in incomplete adjuvant 3-4 weeks apart. A final boost of about the same or somewhat higher dose in aqueous solution i.p. should be followed by fusion 3-4 days later using spleen and draining lymph nodes. To prepare antigen absorbed to alum, make the antigen solution in 10 vols 0.2M aluminium potassium sulphate (pH is about 3). Add 4.5 vols 1M NaHCO₃. Leave 15 min R.T. and centrifuge at 300 g, 15min. Discard supernatant and wash the precipitate 3 times in PBS. B. pertussis vaccine (1-2*10¹⁰/ml) may be added to the final suspension. Inject 20-200 micrograms antigen subcutaneously or i.p. and boost as above. The final boost 3-4 days prior to fusion is probably better given in soluble form i.p..

Immunization with intact cells:

These are highly immunogenic and need no adjuvant. Cells should be washed 3 times in PBS and injected i.p. (some researchers also likes some injected i.v. too). Dose can be from 2-50*10⁸. Boosts may be at 3-8 week intervals, and fusion 2-4 days after final boost. Yields may be higher if recipients rested 4-8 weeks prior to final boost (Oi and Herzenberg(1980) in "Selected Methods in Cellular Immunology" B.B. Mishell and S.M. Shiigi, eds., pp 351-372, Freeman,an Francisco). Long immunization schedules seem unnecessary. Typical immunization: 10⁸ cells/ml, 0.1 ml i.v. 0.4 ml i.p.

Myelomas

Most widely used are descendants of MOPC-21 selected for HAT sensitivity and loss of endogenous IgH (P3-NS1-Ag 4-1; called NS-1) or both H and L (X63- Ag8.653 or NS0/1). Also widely used is Sp2/0-Ag-14, which is a total non-producer variant selected from a hybridoma involving fusion of MOPC-21 and BALB/c spleen cells. A variant of Sp2 claimed to have high fusion frequency and cloning efficiency and rapid growth, is termed "FO", Fazekas and Scheidegger (1980) J. Immunol. Methods 35: 1-21.
Rat myelomas currently available are derived from the LOU/C strain. They include Y3-Ag 1.2.3 (Y3), which is an azaguanine resistant derivative of myeloma R210.RCY3 and secretes kappa chains. Others are IR983F and YB2/0. It should be noted though that their success in production of rat-rat hybridomas has been very limited so far.

**FUSION PROTOCOL in brief**

The myeloma should be a reliable line, preferably obtained from a laboratory which produces monoclonal antibody secreting hybridomas routinely.

It should be tested for mycoplasma contamination and found negative.

We use RPMI 1640 medium routinely, supplemented freshly with glutamine, pyruvate, 3x10^{-5}M 2-mercaptoethanol, Penicillin and Streptomycin (antibiotics are normally not used when culturing established cell lines). Fetal bovine serum is added to between 8 and 12% in complete medium.

The myeloma (e.g. SP2/0) should be harvested from bulk cultures while in healthy mid-log growth phase, and frozen in many aliquots at 20x10^6/vial (1 to 2ml). Two days before the fusion, thaw a vial and distribute cells in about 30 petri dishes (25ml complete culture medium containing 10-15% FBS). Use regular plastic dishes, not tissue culture plastic. Monitor vigorous growth, feed, divide if necessary, and keep cell densities below about 2x10^5/ml. You will need about 50x10^6 myeloma cells per mouse spleen (10^8 lymphocytes).

The day before the fusion:

- Make up or reserve enough medium.
- Prepare fusion solution:
  - 10g PEG in screw cap 100ml bottle- liquify and sterilize by autoclaving. Let cool a little, but while still warm add:
  - 14ml of 15% DMSO in sterile DME medium (without serum) and mix well. pH should be slightly alkaline (7.2 - 8), pink, but not orange or purple.
- Feed the myeloma cells the night before the fusion (at 1-2x 10^7/ml).

Fusion day:

- Put complete medium and PEG fusion mix at 37°C to equilibrate.
- Harvest myeloma cells into 50ml tubes and let stand.
- With sterile technique, remove spleen from immunized mouse boosted 3 days before. Work quickly and gently. Put spleen in a sieve over a sterile petri dish. Scissor the spleen and gently work cells through with the "wrong" end of a sterile 1ml syringe which should be dipped frequently into serum-free medium.
- Transfer the spleen cells to a 50ml tube in serum-free medium, and centrifuge them at room temp., 3-5min, 1500rpm. At the same time centrifuge the SP2/0.
- Suction off the supernatant, and resuspend in 25ml serum-free medium (collecting SP2/0 into one tube). Take small samples for counting while centrifuging the cells again.
- On the basis of the cell counts resuspend the spleen cells at 10^7/ml. Reserve 3ml of this, dilute it with 3ml complete medium, and keep aside to use as feeder cells.
Mix 10ml of the spleen cells (more or less) with enough SP2/0 cells to give a ratio of about 2:1 spleen:SP2/0 (some go as high as 10:1). Mix and spin down together in a 50ml tube.

Use styrofoam rack to insulate tube and help maintain the 37°C temperature during the next step.

Remove ALL the medium from above the pellet. (Some say loosen the pellet, we don't).

Add 0.5ml of warm PEG solution while using the tip of the pipette to stir it into the pellet. The cells clump. Do not be too vigorous.

Lave the pipette in the tube (cell clumps stick to it) and let tube sit only 2min in the rack.

Add warm complete medium DROPWISE from a 10ml pipette and swirl tube:

\[
\begin{align*}
1 \text{ drop } & \text{ every 10 seconds for 1 min.} \\
2 \text{ drops } & \text{ every 10 seconds for 2 min.} \\
5 \text{ drops } & \text{ every 10 seconds for 3 min.} \\
1ml & \text{ every 10 seconds until 10ml has been added.}
\end{align*}
\]

Clumps should still be visible. Spin down.

Remove supernatant and add 25ml complete medium containing 2% HAT. Resuspend GENTLY so as not to break up the clumps much.

Transfer the cells to a bottle or flask containing 275ml 2% HAT medium and the reserved feeder cells.

Distribute approx. 0.1ml per well in about 30 96-well plates - flat bottomed. An auto-dispenser is useful for this, otherwise one or two drops per well from a 10ml pipette.

Incubate. Check daily for contamination.

Three days later add fresh mouse peritoneal cells (macrophage) at about 2000/well in 0.05ml 2% HAT medium.

One week after fusion start feeding with HT medium.

Feed between 2 and 3 times per week by aspirating the top appr. 0.1ml and adding a drop of fresh medium to each well.

Some naked-eye positives will be seen after about 10 days. This is the beginning of the hard work screening, subcloning and freezing.

HAT (hypoxanthine, aminopterin, thymidine) is used as a selection against HPRT-deficient myeloma cells (lacking the hypoxanthine phosphoribosyl-transferase activity) to permit only the growth of hybrids with normal lymphocytes. Aminopterin blocks the pathway for de novo nucleotide synthesis forcing the cell to use the salvage pathway which depends upon the availability of hypoxanthine (a purine precursor) and thymidine (a pyrimidine precursor) and on active HPRT enzyme. Thymidine kinase is also required in the salvage pathway, so HAT would also select against TK-deficient myelomas.
The wild-type HPRT gene is supplied on the X chromosomes of the normal fusion partner. Post-fusion segregation of chromosomes can result in the loss of wt X chromosome(s) and thus the death of some otherwise useful hybridomas. There are other selection strategies, some designed to overcome this disadvantage of the HAT selection (e.g. Taggart, R.T. and Samloff, I.M. 1983. Science 219:1228-1230), but HAT selection is still widely used.

**PURIFICATION & CHARACTERIZATION OF MABs**

Characterization of a monoclonal antibody includes characterization of its class, subclass and light chain, and characterization of its specificity.

Something is already known of its specificity because of the selection procedure during hybridoma production and subcloning.

An early knowledge of the class of antibody helps in the choice of purification procedure.

The quickest and most sensitive method for determining class, subclass and light chain type is to screen the monoclonal antibodies with a panel of antibodies which specifically bind to each subclass or light chain. The detecting antibodies are tagged, eg. with an enzyme for an ELISA readout. Immunoassays are dealt with later.

Polyacrylamide gel electrophoresis is quick way of obtaining class information, and with mini-gel systems and silver staining methods it can be convenient and quite sensitive. An unpurified sample containing a high titre of the antibody may be denatured in SDS without and with mercaptoethanol (to reduce the disulfide bonds and dissociate the antibody into separate heavy and light chains), and electrophoresed alongside a similar sample which does not contain any monoclonal antibody, and alongside a set of protein molecular weight standards, and preferably some antibodies of known class. After staining the gel, the bands of the MAb or its subunits are usually easily visible, and the sizes of the non-reduced form and of the heavy chain are relatively characteristic of the class. The most common, IgM and IgG are easily distinguished from each other.

Large amounts of monoclonal antibody for purification are usually obtained as large volumes (several hundred ml) of culture medium, or smaller volumes (10ml or so) of high titre ascites fluid or serum from hybridoma growth in vivo (in pristane-preconditioned peritoneal cavity of mouse).

Polyethylene glycol or ammonium sulfate precipitation may be used to concentrate antibody from culture supernatants, but this is often not necessary except for IgM, and further purification must be done.

If antigen is available in quantity, and a suitable elution procedure is found, specific antigen affinity chromatography is an excellent way to obtain high-purity antibody in a single step. The antigen is immobilized on a suitable chromatography matrix (forming an immunoadsorbent), the solution containing the antibody is passed through. Only specific antibody binds (except for a small amount of nonspecific binding of other proteins, and this can be reduced by a careful choice of washing steps before elution), and the antibody is eluted by altering the pH (low or high), introducing chaotropic agents like thiocyanate, or salts like 4M Magnesium chloride.

Other affinity chromatography methods bind the MAb to immobilized anti-Ig, or protein-A, -G, or -A/G and elute Ab by lowering the pH. Proteins A, G, or A/G bind the Fc portions of a range of Ig classes and subclasses from various species. The ranges differ, but overlap, protein-A/G having a broadest range. They are especially useful for purification of IgG antibodies. A recent addition is Protein-L which is specific for mouse κ-light chain, and is useful for purifying recombinant forms.
More traditional ion exchange chromatography is a good purification step for Ig, and size separation by gel filtration usually achieves a good purification of IgM because it is larger (900 kDa) than most other proteins in the mixture.

**FRAGMENT PREPARATION**

The Ig is bifunctional and multivalent.

It is sometimes necessary to remove the Fc portion to prevent binding to Fc-receptors on cells, or to prevent activation of the complement cascade. The divalent (Fab')₂ may be released from some immunoglobulins by proteolytic digestion (usually with pepsin, although other proteases may be used).

If monovalent fragments are required, to prevent cross linking of antigens, a disulfide link in the (Fab')₂ may be gently reduced and alkylated to split it into Fab' fragments, or the Ig may be digested with thiol activated papain to yield one Fc + two Fab fragments.

The methods have been worked out mostly for IgG, and originally for rabbit and human immunoglobulins.

Mouse monoclonal antibodies present special problems, and the most successfully digested is the IgG₁. IgG₂a and IgG₃ are more sensitive to proteolysis, so the reaction must be carefully controlled. Also both Fab and (Fab')₂ are produced in the same pepsin digestion of IgG₂a. IgG₂b does not give F(ab')₂, but one Fab and a Fab/c.

**PRECIPITATION OF Ag-Ab COMPLEXES**

Polyclonal antibody binding to antigen which is sufficiently complex to have at least two non-overlapping epitopes can cross-link antigen to form antigen-antibody complexes large enough to precipitate.

If too little antibody is added, the antigen quickly saturates the binding sites so that each antigen is bound by just one antibody (on average), and no precipitate forms.

If the antibody is in great excess (prozone), each antigen may bound by several antibodies, but each antibody binds only one antigen (on average), so no cross linking occurs, and no precipitate forms.

Somewhere between these extremes is what is called the equivalence point at which the ratio of antigen to antibody produces the maximum cross linking and precipitation.

If the reaction is in a fluid phase, the precipitate may be centrifuged to a pellet, washed, and quantified.

The precipitation may be allowed to form in agar, and may provide further information on antibody specificity and antigen purity in one of the plate techniques:

The principle is simple: cut two small wells in a buffered agar layer, and put antigen in one and antibody in the other. The molecules diffusing towards each other through the gel meet and bind. Ab-Ag complexes build up, diffuse more slowly, become further cross linked, and eventually precipitate in the agar in a line part way between the wells. Visibility of the precipitate is enhanced by a protein stain (e.g. Coomassie). The location of the band of precipitation is determined by the relative concentrations of antibody and antigen. If the antigen sample is a heterogeneous mixture, and the antibody preparation has specificities for more than one, multiple bands will be formed.
An elaboration on this is the Ouchterlony plate technique in which one antibody well is surrounded by several wells in which different antigen preparations may be placed. The precipitation patterns formed can be quite informative with regard antibody and antigen complexity and cross-reactivity. The interpretation of such patterns can be found in one of the reference.

There are also techniques which combine electrophoresis in agarose gel followed by diffusion. You may be interested to read on these methods too.

Such methods, however, are limited to polyclonal antisera and the availability of large enough quantities of antigen to allow visible precipitate formation.

More sensitive techniques use radioactivity, fluorescence, or enzyme reactions to report on the amount and location of antibody (bound to antigen, one expects). Some assays use a tagged form of a well-characterized antigen preparation rather than tagged antibody. many procedures also further amplify the signal by building up an inverse pyramid of molecules bound to the first antibody. E.g. one mouse Ab binds to antigen, but two or more rabbit anti-mouse Ig antibodies bind to that one through identical paired (IgG has two identical H and L chains) and different epitopes (the rabbit Ab is a polyclonal preparation). If the rabbit antibodies have had several biotin groups coupled to each of them, and we add avidin-conjugated alkaline phosphatase (or, even better, a mixture of the tetravalent avidin and biotin-conjugated phosphatase) several enzyme molecules become bound strongly to each rabbit antibody.

Greater resolution and more information is achieved by polyacrylamide gel electrophoresis of protein mixtures followed by detection of the separated antigen by antibody binding to what are called Western blots - the proteins from the acrylamide gel transferred onto a suitable membrane such as nitrocellulose. This procedure allows discrimination between different forms of the antigen (e.g. phosphorylated or proteolytically fragmented) and visibly separates them from proteins which bind the antibody non-specifically.

Of course, wherever possible, one must include two controls: positive (e.g. a well characterized form of antigen) and negative (e.g. a similar molecular mixture but lacking the epitopes recognized by the antibody preparation). For quantitative work one must include a dilution series of the positive control.

**TAGGING ANTIBODIES & OTHER MOLECULES**

A few tags frequently used:

Radioisotope - usually $^{125}$I labelled tyrosine residues in the antibody, or may be introduced by reaction of an iodinated chemical (usually Bolton Hunter reagent) with lysine residues. Monoclonal antibodies may be biosynthetically labelled with e.g. $^{35}$S-cysteine or methionine during hybridoma culture.

Fluorochrome - e.g. Fluorescein, Rhodamine, Phycoerythrin etc.

Enzyme - Alkaline (or Acid) phosphatase, Horseradish peroxidase, β-Galactosidase ...

Biotin - allows cross linking to labelled molecules or enzymes through avidin (streptavidin).

Colloidal gold - Electron dense, useful in EM studies, but there are products suitable for macroscopic detection.

Magnetic beads or particles - good for cell separations and immunoprecipitations.
SYSTEMS FOR ANTIGEN DETECTION AND QUANTIFICATION

Visualization or quantitative determination of precipitated immune complexes is not very sensitive. Radioactive tracer techniques, fluorescent tagging, or enzyme mediated signal amplification can greatly enhance sensitivity and allow diverse techniques for quantification and analysis of antigens. The following principles are employed:

1. Antigen is unlabelled and immobilized. Tagged antibody is allowed to bind, unbound is washed away, and remaining bound antibody is measured through the properties of its tag.

1.1. In the above, the antibody need not be tagged, but its presence may be detected by treating it as an immobilized antigen which is detectable by binding a tagged second antibody (e.g. first Ab is mouse IgG, and second antibody is tagged rabbit anti-mouse IgG)

2. A crude antigen preparation is labelled and in solution. Antibody is immobilized and binds the labelled antigen. Unbound antigen and irrelevant molecules are washed away, and the amount of label remaining is measured. Labelled antigen may be eluted from antibody for further characterization.

3. Pure antigen labelled at known specific activity (label/molecule) is mixed with various amounts of unknown crude unlabelled antigen preparation and allowed to bind to immobilized antibody as in 2. Competition by unlabelled preparation reduces the amount of labelled antigen binding and gives a quantitative measure of antigen content in the test preparation.

Many quantitative immunoassays are performed these days on 96 well microtitre plates (clear plastic 8x12 arrays of wells). The plastic is often chosen for its protein binding properties. Protein antigens, or the antibodies may be adsorbed quite stably to the plastic surface at moderately high pH, and the rest of the surface is then blocked with irrelevant protein. The rest of the assay is performed in the wells by additions, washings, and usually enzyme reactions. Frequently colourimetric readout is on ELISA plate readers. Opaque plates are used when detection is of fluorescent tags or chemiluminescent products.

In the sandwich technique, one antibody is bound to the solid support (e.g. bottom of the ELISA plate), Antigen is allowed to bind to it, and then a second antigen-specific antibody, suitably tagged, is used to detect the amount of bound antigen. Unless one is dealing with a homopolymeric antigen, the two antibodies should recognize distinct and distant epitopes on the antigen so as to avoid interference by one antibody with the binding of the other.

All tests should be set up in (at least) triplicate wells. If you do duplicates and get a high and a low value, you have no idea which one may be bad. The third measurement alerts you to possibly spurious results, gives a better mean and allows estimation of the variance.

RIA: Radioimmunoassays

This name generally applies to any immunoassay in which the detection is through measuring a radioactive component of the system. Antibody or antigen is radiolabelled, or presence of antibody is detected by labelled ligand such as protein A.

E.g. Hormone specific antibody is immobilized. Constant amount of labelled hormone is added along with competing standard dilutions or the test sample containing unlabelled hormone. Unbound labelled hormone is washed away, and bound radioactivity is measured.

E.g. $^{125}$I labelled insulin mixed with antibody. Dextran-coated charcoal binds free insulin, but not antibody-insulin complex. Radioactivity in the supernatant is measured. Alternatively, a second antibody is used to precipitate the first antibody and bound hormone. In this case, radioactivity in
the pellet is measure. Cold (unlabelled) insulin in standard and test solutions added to the labelled insulin compete for binding to antibody, and this reduces the signal. Optimum quantities of Ab and labelled Ag must be determined in preliminary titrations.

See slides for a non-radioactive, chromatographic method for measuring insulin in solutions by competition with fluorescently-tagged insulin.

**EIa or ELISA: Enzyme (linked) immuno(sorbent)-assay.**

Enzyme is conjugated to the primary antibody, or to a second antibody, or antibody is conjugated with biotinyl group and detected by binding avidin (or streptavidin)-biotinylated enzyme complexes. The readout is often the coloured product of an enzyme reaction. Enzyme driven chemiluminescence is also possible. See slides for various ELISA systems.

**WESTERN BLOTTING, dot blotting etc.**

Protein antigen (after separation by chromatography or electrophoresis for example) is spotted onto, or transferred onto nitrocellulose or nylon-based membrane. It binds to the membrane and can be detected by immunostaining. Readout can be coloured bands from enzyme reaction producing insoluble coloured product, chemiluminescence or autoradiography.

**FLUORESCENCE ASSAYS (Cytofluorimetry, FACS)**

The presence of a certain antigen on the surface of cells can be shown by the binding of fluorescent antibody. A fluorescent second antibody might be used, or fluorescent avidin if the first Ab is biotinylated. Antibodies to different cell surface antigens can be tagged with different groups to fluorescence at different wavelengths. This allows multi-colour fluorescence analysis.

**BIOSENSORS** (we did not cover this in class this year - no exam question on biosensors)

New technology is on the market which allows real-time analysis of macromolecular binding interactions. Biosensors are highly sensitive devices in which measurable signal changes are produced in response to changes in biomolecule concentration.

**Surface plasmon resonance detection.**

The BIAcore system (BIA = Biospecific Interaction Analysis) of Pharmacia is based on a phenomenon called plasmon resonance. You need not know the physics behind this, but may follow up the topic, if it interests you, through the following references: M. Malmqvist. 1993 Nature 361:186-187; Jönsson et al. 1991 BioTechniques 11:620-627.

It is based upon changes in the direction of minimum light reflection from a multilayer surface of gold coated glass, immobilized receptor, and binding ligand. More or less ligand bound to the surface increases or decreases the refractive index of that layer, and alters the reflected light interference pattern.

**Piezoelectric detection systems.**

Piezoelectric crystals have resonant frequencies which are sensitive to changes in the mass load on a crystal face. With a receptor immobilized to one face of the crystal, changes in the amount of ligand bound to the receptor molecules alter the resonant frequency. Another property - piezoresistance - is used to measure minute forces produced by a magnetic field on a ferromagnetic particle coupled to the antibody.
RECOMBINANT ANTIBODIES

The technology has been developed for expressing heavy and light chain variable domains in a chimeric fusion protein on the surface of E. coli filamentous phage in sup E hosts. Transferal of the gene into a bacterium lacking an amber suppressor gene causes secretion of a soluble form of the so-called ScFv (single chain fragment variable) proteins into the periplasmic space. Pharmacia is now marketing this technology (see Pharmacia publication "Analects" vol. 22(4) autumn 1994).

Using recombinant techniques, Fv may be obtained fused to practically any other useful protein domains.

OTHER IMPORTANT MATERIAL COVERED - see the “slides” file.

References (somewhat old, but still useful list. See also the technical publications from companies which supply reagents and equipment for immunochemistry).

Here are a few sources of information relevant to this class. There are certainly others, some more recent, in the library. You will undoubtedly find some useful e-books available to McGill. Check it out on MUSE when you need to go further into the systems and techniques.


Handbook of Experimental Immunology 1: Immunochemistry. D.M. Wier ed. Blackwell Scientific Publications, 4th edition. (QW 504 H236 1986 v.1) (There is probably a more recent edition by now. Ask in the library, or ask the publisher if they have a more recent alternative. This was a very good source of information in its day, and much of it will still be useful).

Gregoriadis G. "Immunological adjuvants: a role for liposomes) 1990 Immunology Today 11:89-97 (also check out literature from companies re. Hunter's TitreMax or the RIBI product)

Current Protocols in Immunology. John Wiley and Sons Inc. (continually revised and expanded through supplements; also available in CD-ROM version; overpriced, but good lab. book)