Basic Immunology

Production of anti-peptide antibodies exploits the ability of an animal to protect itself from infection.

Macrophages engulf and break down foreign antigens. Also known as Antigen Presenting Cells (APCs), these cells degrade foreign protein into peptides, which are then presented on the surface of the cells after having been complexed with Major Histocompatability Complex (MHC) class II protein. Helper T-Cells bind the APC, and these cells have a receptor that recognizes when the MHC class II proteins have bound peptide. Each Helper T-Cell receptor is highly specific for the MHC class II protein/ bound peptide shape, and if the Helper T-Cell receptor can bind the complex, Interleukin 2 is secreted by the cell which results in proliferation of that class of T-cell.

At the same time as this is happening with the T-cell, The B-cells are also engulfing and degrading the antigen, but these cells have an antibody like receptor that recognizes the particular epitope, The B-cells then present the antigen fragments in a similar way to the APCs.

When a Helper T-cell binds via its receptor to the B-Cells MHC II peptide receptor, an antibody response ensues. After binding, the Helper T-Cell secretes Interleukin-4 and -5, which cause the B-Cells to differentiate into plasma and memory cells. It is the plasma cells that secrete the antibodies whilst the memory cells retain the surface antibody, and are able to mount a more rapid response in the event of being challenged with the same immunogen again (the so-called secondary response). It is this secondary response, which produces far more antibody than the initial challenge that will be exploited for our purposes.

If the antigen is too small, or the presented antigen fragments are not recognized by a Helper T-cell, there will be no immune response.

Peptide Design

The first thing that one has to consider when selecting a peptide to direct antibody synthesis, is its ultimate use, namely which region of the protein you wish to raise the antibodies. The sort of example could be to follow protein processing, or phosphorylation of certain sequences on the protein. However, whilst antibodies raised against peptides will recognize the peptide, it does not always follow that the antibody directed against the peptide will recognize the native protein because the synthetic peptide may not sufficiently mimic the parent protein. Consequently, as will be mentioned later, the cross reactivity of the antibody will have to be determined. The epitope, the antigenic determinant, defined as a region of six to twelve amino acids or carbohydrate residues to which an antibody can bind, must be available for the antibody to bind which means that the three dimensional state of the protein is important. In addition, antibodies can be raised against linear stretches of the protein (so called continuous epitopes) or against nonlinear parts of the protein that become juxtaposed in the folded state (so-called discontinuous epitopes). A discontinuous epitope can also be created when two polypeptide chains come together. Anti-peptide antibodies directed against continuous epitopes are quite often successful, whereas when directed against discontinuous epitopes the outcome is not so certain.

Potential antigenic determinants tend to be from sequences that are exposed (on the surface of the molecule), hydrophilic and flexible. This is because most proteins have their hydrophobic residues buried in the interior of the molecule. Consequently, peptide antibodies directed against the N- or C-terminal of a protein are often a good choice because being at the ends they are both flexible and charged (because of the amino group of the former, and the carboxyl group of the latter). With the C-terminus peptides, care should be taken that the sequence does not form part of a transmembrane region, whilst with N-terminal derived peptides, particularly when the peptide sequence is derived from a cDNA, care should be taken not to include any signal/leader sequence. Where possible software packages should be used to determine the hydrophylicity of the native protein.

When it comes to discussing optimal peptide length, there are two ways of looking at the problem. Longer peptides (up to 40 amino acids) could be better in that they could include multiple epitopes, although they could be difficult and expensive to synthesize. Short peptides (e.g. seven amino acids) may make the peptide antibodies so specific that the may not recognize the native protein at all, or if so, only weakly. Most researchers choose peptides of between 12 and 20 amino acids in length, and because of this, many peptide-producing companies offer discounts within this range.

Peptides by themselves are too small to be immunogenic, and so need to be coupled (see section about coupling) to other molecules in order to stimulate an immune reaction. Because of this, and the desirability of keeping the peptide structure as close to its natural state with regard to orientation and net charge, it follows that if an additional residue is added to assist coupling (see later), care must be taken to ensure that this residue is placed at the opposite end of the epitope. So when considering N- and C-terminal peptides, the former should have this linking residue on the C-terminus, and the latter should have the residue on the N-terminal side. If no linking residue is to be added, then the C-terminus should be blocked with an amide group in the case of an N-terminal peptide, and with a C-terminal peptide the N-terminal end should be blocked by addition of an acetyl group. It is important to note however that the "natural" ends should not be blocked in order to try and maintain the epitopes' native state. For

peptides derived from an internal part of the protein, an additional residue for conjugation can be placed at either end of the molecule. With such peptides the C-termini should be amidated, and the N-terminal acetylated.

As an alternative to coupling a peptide to a carrier protein to induce immunogenicity, peptides can be synthesized as multiple antigenic peptides (MAPs). These are peptides that are multiply linked, and as such do not require linking to a carrier protein which may help to obviate the problem of raising antibodies to the latter.

Synthesizing Peptides

However not all peptides are easy to synthesize or subsequently handle. Peptides with a high number of hydrophobic amino acids such as Ile, Leu, Met, Phe, Trp and Val will be most likely insoluble in aqueous solutions. It is advisable to keep the number of hydrophobic residues in a peptide to below 50%. In some cases it can be possible to add hydrophilic sequences to one end of an otherwise quite hydrophobic peptide in order to improve the overall aqueous solubility.

Peptides containing multiple residues of Cys, Met or Trp can be problematic because they are susceptible to oxidation and/or side reactions. Also, with cystein, its very presence would indicate some special conformation in the protein, because of sulfhydryls and associations with Zinc or other divalent metal cations at that point that would not be mimicked in the peptide. This would raise the possibility that any antibodies produced might recognize the peptide only and not the native protein. Some of these problems can be obviated by substitution with conservative replacements: -

- Norleucine can often be used instead of Met.

- Ser can often be used instead of Cys

Finally if beta-sheet formation occurs during synthesis of the peptide, deletions are likely to result. These problems can be avoided if the sequences do not contain multiple or adjacent residues of Gln, Ile, Leu , Phe, Thr, Tyr or Val. Again, conservative replacement residues can be used if there is no other alternative:

- putting Gly or Pro every third residue

- replacing Gln with Asn, or Thr with Ser.

Finally, but no less importantly, the sequence should be looked at to decide which coupling method would be the best one to use. This will be discussed in the next couple of sections.

Peptide Coupling

Peptides are too small to generate an immune response by themselves and therefore need to be coupled to larger molecules in order to become immunogenic. This is because the antigen needs to be sufficiently large in order to be recognized by the Antigen Presenting Cells (macrophages). Also, protein carrier molecules have sequences that are Helper T-cell epitopes and therefore help Helper T-cells to proliferate and interact with the B cells. This is essential if there is to be a strong immune response. As the carrier protein is chosen to be "foreign" to the animal in which the antibodies will be raised, the carrier protein will enhance the immune response.

Which Carrier Protein to use?

The choice of which carrier protein to conjugate to will depend on the animal to be used for immunization. Proteins that are least similar to the host animal proteins will generate the best immune response. It is important to remember that antibodies will also be made against the carrier protein as well as the peptide, so this may have consequences when using the antibody.

Therefore it is best to avoid using a carrier protein that features in the assay in which the antibody will be used. Keyhole Limpet Haemocyanin (KLH) and Bovine Serum Albumin (BSA) are the two carrier proteins most commonly used, but ovalbumin Rabbit Serum Albumin and Tetanus toxoid have also been used. Here are a few individual comments about them:

Keyhole Limpet Haemocyanin (KLH)

This protein comes from the mollusk Megathura crenulata and because of its source, it is a good carrier when used in rabbits or mice. As a protein, it consists of 5 subunits, which exist in 5 aggregate states at pH 7.4. At pH 8.9 though, it is completely disaggregated. It has the advantage of having many Lysine residues

available for coupling, a number that increases as the subunits dissociate. KLH can be difficult to handle due to its poor

solubility in water. A common problem in this regard is that it can on occasion precipitate during the cross-linking.

Bovine Serum Albumin (BSA)

This is more water-soluble than KLH, and as BSA is often used as a blocking agent in immunochemical assays. If the assay system for the antibody contains BSA it should not be used as the carrier since antibodies will also be raised against BSA.

Ovalbumin

Often used as a second carrier protein to test whether the antibodies are specific for the peptide and not the carrier.

Rabbit Serum Albumin (RSA)

This could be a choice to take when immunizing rabbits if a response to the carrier protein is kept to a minimum.

As a concluding point, when screening by ELISA one must also include a conjugate where the peptide has been made against another carrier protein to eliminate false positives made by antibodies to the carrier. The alternative here is to coat the plate directly with unconjugated peptide.

Conjugation Methods

Most of the coupling methods utilize either free amino groups (alpha amino or Lysine), sulphhydryl groups (Cys), or carboxylic acid groups (alpha carboxyl, Asp and Glu) It should be born in mind that the peptide sequence chosen should not have multiple stretches of amino acid residues that react with the coupling method of choice (unless they are at either the desired C-or N-terminal end.

The Carbodimide Method

The 1-ethyl-3-(3-dimethylaminopropyl) carbodimide hydrochloride (EDC) activates the side chain carboxylic groups of Asp and Glu as well as terminal carboxyl groups so that they can react with primary amines. If the carrier is activated first the carrier protein will be coupled through the N-terminal alpha-amine or through a Lysine if it is present in the sequence.

It can be used with peptides containing N-terminal amines and C-terminal carboxyl groups. This method should not be used if the epitopic sequence contains Asp, Glu or Lys.

The MBS Method

The m-Maleimidobenzoyl-N-hydroxysuccinate ester (MBS) links carrier protein to the peptide through a Cys residue. If the Cys residue is put onto one end of a peptide for this purpose, a very specific link is made. However, for peptide synthesis purposes it is better that the additional Cys residue is on the N-terminus. If the epitopic sequence of the peptide contains a Cys or a Lys this method should be avoided.

The Gluteraldehyde Method

Gluteraldehyde links compounds through the amino groups of Cys His and Tyr. It is very reactive, which has the drawback of yielding ill-defined conjugates (which may nevertheless be highly immunogenic), but the method can prove useful when the only free amino group on the peptide is at the N-terminus. Like the MBS method, should the epitopic sequence contain a Cys or a Lys, then this method is best avoided.

Choice of Animals

In this case, it is important to choose an animal in which the antibody will be raised that is as distantly related as possible to the source of the protein. This will make it more likely that the protein is recognized as foreign, and that antibodies against it will be made.

Adjuvants

If a strong antibody response to the antigen is required, then it is essential that an adjuvant be used. Adjuvants are non-specific stimulators of the immune system, but how they actually work is not entirely known. However they are thought to play a multifunctional role. It may be that they slow down the rate of release of the antigen from the site

of injection, which is known to be beneficial for a good immune response. Also. the adjuvant may protect the antigen from premature breakdown, It could also be that adjuvant stimulates the release of lymphokines which would increase the activity of the macrophages (APC cells)

There are many kinds of adjuvant, the most famous of which (historically speaking) is Freunds Complete adjuvant, which is made up of mineral oil and killed mycobacterial cells. (Freunds incomplete adjuvant lacks the killed cells). However, the toxic nature of this adjuvant has led to its use being barred in some countries and in turn has stimulated the development of alternatives, such as produced by RIBI, Titer Max and many others.

Duration of Immunization Protocols

Standard protocol 94 days

However it should be noted that a response to a peptide may take six months to develop, whereas with proteins this is the reverse-the response may diminish with time. Bear this in mind when deciding whether or not to abort the project

Additional note: A protein may become more antigenic if it is boiled prior to mixing with the adjuvant.