Review article

Strategies for mapping and imitating viral B-cell epitopes

John Cason *

*The Richard Dimbleby Laboratory for Cancer Virology, The Rayne Institute, St Thomas' Hospital,
Lambeth Palace Road, London SE1 7EH, UK*

Abstract

Identification of viral B-cell epitopes is of importance in the selection of peptides for inclusion
into subunit vaccines, the development of virus-specific serological tests and understanding the
interaction of antibodies with viruses at a molecular level. B-cell epitopes can often be determined
unequivocally by X-ray analyses of antibody–antigen complexes. This technique is, however,
time-consuming and alternative strategies have now been developed for identifying epitopes. This
article provides an overview of approaches which are currently available for mapping and
imitating B-cell epitopes.

Keywords: Virus; Epitope mapping; Antibody

1. Introduction

Viral infection usually results in the production of specific glycoproteins (antibodies,
assembled from four constant and four variable polypeptide chains) by the host's
B-lymphocytes. The variable regions of antibodies form two receptors (paratopes) which
are able to bind, predominantly via hydrogen bonds, to discrete regions (epitopes,
antigenic determinants) of a foreign agent and often promote its elimination from the
body. Whilst a concise definition of what constitutes an epitope is a contentious issue,
for the purposes of this paper an (proteinacious) epitope is presumed to comprise of
those amino acids of an antigen which are involved in bond formation with residues of a
corresponding paratope.

There are also conflicting views upon how a paratope actually gains access and binds
to an epitope. As reviewed by Van Regenmortel (1989), some believe this interaction

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* Corresponding author.

may be viewed as a 'lock-and-key' system (whereby a paratope recognizes a fixed region of an antigen which has a directly complementary shape or chemical characteristic). Others envisage an 'induced-fit' model, such that antibody binding is dependent upon slight movements of side chains, or backbone, of the antigen to accommodate antibody (i.e., akin to the adaptation in shape necessary to position a hand inside a glove).

What is clear is that during a natural infection the vast majority of antigen-specific antibodies are directed against conformation-dependent (assembled) epitopes present on native structures (Laver et al., 1990). That is, epitopes which depend upon inter- and/or intra-molecular interactions which permit distant (in terms of linear sequence) amino acids to come into close proximity to form an antigenic determinant. It is, however, equally apparent that another type of epitope also exists which bears a much closer resemblance to a protein's primary structure. Such linear (or continuous) epitopes consist of five to eight contiguous residues of a protein's amino acid sequence. This division between conformation-dependent and linear epitopes may be somewhat artificial in the case of antibodies induced by an intact protein, since any linear epitopes recognized may represent constituent parts of conformation-dependent epitopes.

Antibodies to linear epitopes are of considerable value as they are easy to prepare. Many workers have used denatured proteins or synthetic peptides (i.e., both essentially

Table 1
Some requirements and characteristics of different epitope mapping strategies

Method	Live	Crystal	Pure	Sequence	FSR	Epitope type		Actual epitope sequence *
						C	L	
(i) antigen is readily available:								
X-ray cryatallography	−	+	+	+	−	+	? +	+
Escape mutants	+	−	−	+	−	+	? +	+
Proteolytic cleavage	−	−	+	+	−	−	+	+
(ii) Antigen is known but protein is unavailable:								
Recombinant protein subregions	−	−	−	+	−	−	+	+
BAL-31 truncated recombinant proteins	−	−	−	+	−	−	+	+
Synthetic peptides	−	−	−	+	−	−	+	+
(iii) The antigen is unknown:								
Peptide mimotopes	−	−	−	−	−	+	+	−
Combinatorial libraries	−	−	−	−	+	+	+	−
Random peptides	−	−	−	−	+	+	+	−

+: particular characteristic required or, −: not; Live: live virus/bacteria and host indicator system (cell culture or animal) necessary; Crystal: antibody/peptide required in pure crystalized form; Pure: Purified native protein desirable; Sequence: DNA sequence details a prerequisite; FSR: further amino acid or DNA sequencing needed in order to identify the epitope; C: ability to mimic conformation-dependent or, L: linear eptitopes.
* Whilst all mapping techniques produce a defined epitope, those techniques dependent upon the derived or random deduction of an epitope may not identify a sequence actually present in the original protein immunogen.

linear immunogens) to produce specific antisera or monoclonal antibodies (mAb) in experimental animals. Such antibodies are useful as molecular probes and can sometimes have biological activity. Identification of linear immunogens that can induce neutralizing antibodies to intact virus particles may be of considerable interest to those concerned with the design of subunit viral vaccines.

Currently, sequence data of medically important antigens are being accrued at a hitherto unprecedented rate and it is now relatively easy to produce recombinant DNA-derived antigens and their corresponding antibodies. The established method of epitope mapping, X-ray crystallography, suffers from being relatively slow. Accordingly, the techniques of molecular biology and organic chemistry have been utilized to develop strategies for identifying antigenic determinants. Most approaches are currently biased toward the identification of proteinacious epitopes on viruses, due to extensive pre-existent knowledge of protein chemistry and the limited size of viral genomes (which facilitates rapid sequencing and deduction of potential antigens). Here, techniques for mapping epitopes are reviewed and subdivided into those applicable to three scenarios, namely where native viral antigen is: (1) characterized and readily available, (2) known, but unavailable and, (3) completely unknown. Some of the relative merits of the different epitope mapping methods described below are summarized (Table 1).

2. Epitope mapping strategies

2.1. Antigen is available

2.1.1. X-ray crystallography

The structure of conformation-dependent epitopes can be often be elucidated with certainty by X-ray crystallographic studies of antigen-Fab (Fab: the Fragment antigen binding of an antibody) complexes (Laver et al., 1990). This approach permits detailed analyses of antigen-antibody interactions and has been used to solve the three-dimensional structure of an idiotype/anti-idiotype complex (Bentley et al., 1990) and an epitope on rotavirus (Prasad et al., 1990). However, X-ray crystallography is expensive, slow, restricted to specialized departments and does not always provide concise results: for example the neutralizing epitopes on foot-and-mouth disease virus could not be clearly established by this technique (Parry et al., 1992). Furthermore, on occasions antigen-Fab crystals can prove difficult – if not impossible – to prepare. Despite these problems X-ray crystallography, in comparison to reductive epitope mapping methods (below), is believed to provide the most accurate view of epitope/paratope interactions, since it permits the topographical localization of epitopes in the context of an intact native antigen.

2.1.2. Escape mutants

Infectious agents can be investigated for their ability to mutate after exposure to a neutralizing antibody (in vitro or in vivo). The rationale behind this approach is that any nucleic acid changes which result in amino acid coding differences between an escape mutant and the original isolate contribute to an epitope recognized by a neutralizing

Table 2
Some proteases of use for epitope mapping

Enzyme	Cleavage site	
	NH$_2$	COOH
Trypsin	(............Lysine---X..........	
	(..........Arginine---X..........	
Chymotrypsin	(.....Phenylalanine---X..........	
or	(........Tryptophan---X..........	
Pepsin	(..........Tyrosine---X..........	
Thermolysin	(.................X---Leucine.....	
	(.................X---Isoleucine..	
	(.................X---Valine......	
Cyanogen bromide *	........Methionine---X..........	
V8 protease	(.....Glutamic acid---X..........	
	(.....Aspartic acid---X..........	
Lysine protease	(...........Lysine---X..........	
Clostripain	(..........Arginine---X..........	
Papain	(...........Lysine---X..........	
	(..........Arginine---X..........	
Elastase	Carboxylic-side of non-aromatic neutral residues.	
Ficin	(...........Lysine---X..........	
	(.........Arginine---X..........	
	(..........Leucine---X..........	
	(..........Glycine---X..........	
	(.........Tyrosine---X..........	

Form: Drapeau et al., 1972; Lehninger, 1976; Boehringer, 1991.
X: any amino acid; * non-enzymic cleavage.

antibody. This method is particularly useful for studies of RNA viruses as they have no proof-reading functions, which results in greater variation in RNA genomes (whilst DNA viruses may have a similar propensity to mutate any changes are corrected). Interpretation of such results may conceivably be complicated by the occurrence of random mutations at sites which form no part of a neutralizing epitope. Nevertheless, this technique has successfully been used to define neutralizing epitopes of poliovirus (Minor et al., 1985, 1986), foot-and-mouth disease virus (Xie et al., 1987) and avian bronchitis virus (Kant et al., 1992).

2.1.3. Proteolytic cleavage

Protein antigens can be dissected using enzymes that cut proteins between specified amino acid residues (e.g., Table 2) to produce fragments whose molecular weight can be calculated from the protein's amino acid sequence. By subjecting the resultant polypeptides to polyacrylamide gel electrophoresis and Western blotting, epitopes can be localized on an intact protein from the apparent molecular weight of immunoreactive fragments. Analyses of such proteolytic cleavage products have led to the identification

Table 3
Some expression systems available for producing antigens from recombinant DNA

	System	Epitopes	Availability	Yield	Comments
P	E. coli (e.g. pGEX, pEX, pATH) *	L	Com	H	Easy to use, high yield, many plasmid vectors available.
E	Insect cells (via baculovirus)	C, L	Com	H	Many plasmid vectors available, can form VLP.
E	Yeast	C, L	Com	H	Can form VLPs.
E	Mammalian cells (via vaccinia virus)	C, L	–	L	Complex as different promoters have differing stop codons: VLPs.
E	Simian cells via EBV	C, L	–	L	
	Cos-7	C, L	–	L	
	CV1	C, L	–	L	
E	Murine C127 [via BPV]	C, L	–	L	
	fibroblasts 3T3 [via retroviruses]	C, L	–	L	
E	Xenopus oocytes	C, L	–	L	
E	Rabbit reticulocytes	C, L	Com	L	

P: prokaryotic or, E: eukaryotic expression system. Epitopes, L: predominantly linear, C: conformation-dependent. VLP: virus-like protein structures. Com: various kits and vectors available commercially; * common plasmids for expression include pEX produces a β-galactosidase fusion protein; pATH: produces a tryptophan-E-synthetase fusion protein; pGEX: produces a glutathione-S-trnsferase fusion protein; EBV: Epstein-Barr virus; BPV: bovine papillomavirus.

of group-specific antigenic determinants on the major capsid protein of group A rotaviruses (Kohli et al., 1992). It should, however, be noted that this and other (see below) similar epitope mapping techniques are limited in that what is measured is actually the cross-reactivity of an antibody (to a complete protein) with polypeptide fragments. It is purely an assumption that such fragments assume structures even similar to those which they adopt in the context of their position within an intact protein.

2.2. Antigen is known but protein is unavailable

DNA or RNA coding for an otherwise unavailable (or even unknown) protein antigen can usually be amplified, cloned and sequenced. Recombinant proteins and protein sub-regions may then be constructed for use in epitope mapping studies. The most striking example of the effectiveness of this strategy is the isolation, characterization, and development of a serological test for a previously unidentified pathogenic virus, namely hepatitis C (Choo et al., 1989; Alter et al., 1989).

2.2.1. Production of recombinant protein antigens

Nucleic acid coding for a protein can be cloned into one of a variety of prokaryotic or eukaryotic expression vectors, many of which are now available commercially (Table 3). The most easily manipulated and commonly used systems are those which involve expression of proteins in E. coli recombinant plasmids. Such expression systems are

advantageous, as large quantities of recombinant protein can be prepared quickly and cheaply. However, other systems may prove to be more appropriate if, for example, it is suspected that an epitope may be influenced by post-translational modifications such as glycosylation, phosphorylation or myristilation. In such a case, eukaryotic expression via the commercially available baculovirus/insect cell or yeast kits, permit the production of high levels of post-transcriptionally modified viral proteins which can sometimes self-assemble to form virus-like particles (Carter et al., 1991; Kirnbauer et al., 1992).

Panels of subregions of a protein antigen can be produced from restriction endonuclease digests of coding DNA in order to map B-cell epitopes. Such recombinant protein subregions have been used to explain serological differences between strains of human cytomegalovirus (Basgoz et al., 1992). Alternatively, proteolytic cleavage (above) can be employed to determine the position of epitopes, though this may be complicated if fusion proteins are used. Another approach is to digest an antigen's coding DNA with *BAL*-31 exonuclease (which selectively cleaves nucleotides from the 3′ terminus) and remove samples at intervals prior to insertion into a plasmid. This permits the production of a panel of polypeptides of varying length (successively truncated from the carboxylic terminus) which can be used to screen for an immunoreactive region. DNA coding for this region can then be sequenced and an epitope determined. Use of *BAL*-31 deletion mutants of the VP2 protein of human rhinovirus type-2 have resulted in the identification of a neutralizing epitope (Skern et al., 1987).

2.2.2. Synthetic peptides

With only a rudimentary knowledge of organic chemistry it is possible to construct small peptides corresponding to subregions of a protein antigen using f-moc or t-boc chemistry. Peptides are often synthesized as a series of nested 15-mers with a 5 amino acid overlap, so as to encompass the entire protein under investigation. Peptides can then be used as antigens in enzyme-linked immunosorbent assays (EIA) to deduce an epitope: this approach is expensive but has been used to define a type-restricted epitope on the major coat protein of human papillomavirus type-16 (Cason et al., 1989). The development by Geysen et al. (1987) of the pin system (PEPSCAN) has revolutionized this form of epitope mapping as it permits the rapid and cheap construction of hundreds of peptides. Briefly, peptides are constructed on polyethylene pins which are then used in situ as antigens in EIAs. Whilst peptides are generally considered to be primarily of value for the identification of linear antigenic determinants, recent work has shown that aggregated peptides may also be able to imitate conformation-dependent epitopes (Rao et al., 1992).

Having identified an epitope the relative contribution of each of its amino acids to antibody binding can be investigated by synthesizing artificial mutant peptides. For a hexameric epitope, a set of 120 peptides may be produced in which each constituent amino acid is sequentially replaced by the remaining 19 possible amino acids whilst keeping the other five amino acids unchanged (Geysen et al., 1987). Similarly, a minimum binding site for an antibody can be identified by creating panels of peptides sequentially truncated from the amino and/or carboxylic terminus. Synthetic peptides have been employed to localize antigenic determinants recognized by neutralizing mAbs to foot-and-mouth disease virus (Meloen et al., 1987), rhinovirus-14 (Sherry et al.,

1986), human immunodeficiency virus (Papsidero et al., 1989), murine coronaviruses (Luytjes et al., 1989) and a type-restricted epitope on human papillomavirus type 16 (Cason et al., 1989). Whilst peptide mapping techniques are particularly useful for identifying epitopes recognized by mAb or polyclonal antisera derived from hyperim-mune animals, the application of this technology for identifying epitopes recognized by antibodies in human sera can often prove to be problematic due to high levels of antibody binding to peptides per se.

Synthetic peptides to selected regions of a protein can also be used as immunogens to raise epitope-defined antibodies. Such anti-peptide probes can then be used to investi-gate the expression, or biologic function, of a particular epitope on a native protein or virion. This method has been used to study the neutralizing activity of mAbs to selected peptide subregions on human immunodeficiency virus (Langedijk et al., 1991), and antibodies to certain linear B-cell epitopes have neutralizing activity for foot-and-mouth disease virus (Doel et al., 1988), influenza virus (Muller et al., 1990) and bovine papillomavirus type-2 (Cason et al., 1993).

2.3. The antigen is unknown

Situations can arise when a mAb of interest is obtained using a crude mixture of proteins such as a cell homogenate from a clinical lesion. Whilst the original immuno-genic protein may be unknown, epitope mapping strategies have been devised which are independent of any prior knowledge of an antigen's characteristics. In practice these techniques involve either presenting a large number of peptide shapes to the antibody under study, or deriving an epitope mimic (mimotope) by a process of iteration.

The spectrum of shapes capable of acting as epitopes is limited by size since – by definition – they must be able to fit into the corresponding paratope. The repertoire of shapes is further reduced by the host's down-regulation of autoreactive paratopes and also by the acquisition of tolerance to other determinants. Despite these restraints, an individual animal is still thought to be capable of producing specific antibodies to about 10^8 unique epitopes (Roitt et al., 1985).

As noted above, the majority of antibodies are usually directed against conformation-dependent epitopes. However, there is no reason to believe that random peptides are unable to adopt shapes that are similar – or even identical – to conformation-dependent and linear epitopes. The approximate number of residues of an epitope in contact with a paratope is about six amino acids, thus if one creates a random-sequence hexameric peptide library comprising the 20 amino acids coded for by DNA (i.e., the 1-stereoiso-mers), some 6.04×10^7 unique amino acid sequences may be generated. If it is assumed that each of these sequences forms an unique three-dimensional shape, random hexam-eric peptides may mimic approximately 60% of the epitopes recognized by the B-cell repertoire. Such approaches may be advantageous, since it may also be possible to mimic non-proteinacious epitopes.

2.3.1. Combinational libraries

As it has been shown that antibodies are capable of reproducibly binding to dipeptides, a bank of antigenic shapes can be prepared by constructing just 480 peptides. The first two amino acids of a hexamer are defined by constructing 400 peptides which

contain all possible amino acid combinations of the first two positions, whilst the remaining four positions are synthesized using an equimolar mixture of all 20 amino acids. This panel of peptides is then screened using the antibody under investigation and the most reactive defined dimeric sequence is retained. The third amino acid position is varied for all 20 amino acids and the remaining 3 residues are produced from an equimolar mix of all amino acids. After rescreening and deduction of the most appropriate third amino acid, the 20 variants of the fourth position are tested and one selected and so on, until the complete optimal-binding hexameric epitope is derived (Houghten et al., 1991).

2.3.2. Random peptide libraries

Random pentameric peptide sequences are synthesized onto small resin beads, so that each bead is coated with a single peptide species. This is achieved by coupling 19 amino acids (cysteine being omitted to eliminate disulphide cross-linking) individually to 19 aliquots of beads. The aliquots are mixed, redivided into 19 samples, the second amino acid coupled and the process repeated until a pentapeptide library is completed. In this way a library of 19^5 (2.47×10^6) random peptides can be produced. This method has the advantage that an individual bead should bear only a single peptide species. Antigenicity of peptide-coated beads can then be identified by a localized colour reaction by EIA upon microscopic examination. Immunoreactive bead(s) are then retrieved, washed free of antibody with urea, and the amino acid sequence of the antigenic determinant/antigen mimic can be identified using a microsequencer (Lam et al., 1991).

Random peptides may also be expressed using a fd vector which is related to the M13 family of filamentous bacteriophages. DNA encoding a peptide can be introduced between the two domains of the pIII protein (Smith, 1985) or, near the 5′ terminus of pIII (Parmley and Smith, 1988). Short oligonucleotides encoding peptide sequences inserted at these regions are expressed on the phage surface. By inserting random oligonucleotides coding for peptide sequences of 6 to 15 amino acids, it is possible to generate libraries of recombinant phages which express small random amino acid sequences on their surface (Scott and Smith, 1990; Cwirla et al., 1990). Samples of individual colonies can be transferred to nitrocellulose sheets and tested with the antibody under investigation by a dot-blot assay. Colonies expressing the appropriate epitope can then be identified and, after DNA sequencing, the amino acid sequence derived.

A potential problem associated with the random peptide-based approaches described above is that it is not uncommon for an antibody to bind to epitopes other than the one against which it was originally raised, a phenomenon known as heterospecificity or heterocliticity (Makela, 1965; Al-Moudallal et al., 1982; Harper et al., 1987). Hence, it is possible to identify antigenic determinants whose amino acid sequence does not occur in that of the original protein immunogen. Furthermore, as these techniques have not proved popular it is presently difficult to assess how effective they actually are.

2.3.3. Mimotopes

As noted above, antibodies bind reproducibly to dipeptides, thus it is possible to select (from all 400 possible dipeptides) one dimer which is best recognized by a mAb.

To increase the spectrum of antigenic shapes which can be imitated in a given peptide unnatural amino acids (i.e., D-stereoisomers) can also be included. For an identified dipeptide 'epitope', all four possible combinations of L- and D- (the unnatural stereoisomers) amino acids for a dipeptide can be tested and the sequence exhibiting highest mAb binding selected. This derived dipeptide can then be tested after the addition of each of the 40 possible D- or L- amino acids added to the carboxylic or amino terminus (with, or without, alanine spacers) to determine the optimal third and fourth residues. The resulting 4-mer peptide is then selected as the mimotope (Geysen et al., 1986, 1987). Again this epitope mimicking approach has not proved popular amongst experamentalists and its value is hence difficult to evaluate.

3. Summary

This article provides an overview of the approaches currently available for the identification of B-cell epitopes. At present the newer (reductive) B-cell mapping techniques appear to be very effective for mapping epitopes recognized by mAbs or polyclonal antisera raised against linear proteinacious epitopes – an obvious limitation. Whilst the random peptide and mimotope approaches may be of value for deriving mimics of conformation-dependent epitopes, new techniques are required so that this form of antigenic determinant can be mapped with accuracy. One possible answer could involve the generation of large numbers of mutant proteins to localize those amino acids, which are crucial for antibody binding. Advances in molecular modelling techniques using computer graphics may also enable the identification of an antigenic determinant on a sequenced protein which is recognized by a (DNA sequenced) mAb paratope.

Another area which remains relatively unexplored is that of mapping epitopes on non-proteinaceous antigens: this will no doubt progress in parallel with advances in sugar and lipid biochemistry. Paradoxically, peptide strategies for mapping B-cell epitopes, as described above, are now realized to be most appropriate for the identification of T-cell epitopes, since these are to consist of short peptides rather than conformation-dependent structures. Indeed, the PEPSCAN method has now been modified to permit cleavage of peptides from polyethylene pins so that they can be used to determine T-helper and T-cytotoxic cell determinants on a foreign protein.

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