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Isolation of sequences from a random-sequence expression library that mimic viral epitopes

Johannes A. Lenstra [a], Joannes H.F. Erkens [a], J.G.A. Langeveld [b], Willem P.A. Posthumus [b], Rob H. Meloen [b], Fátima Gebauer [c], Isabela Correa [c], Luis Enjuanes [c] and Keith K. Stanley [d,1]

[a] *Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht, Netherlands,* [b] *Central Veterinary Institute, Lelystad, Netherlands,* [c] *Centro de Biología Molecular, Universidad Autónoma, Madrid, Spain, and* [d] *European Molecular Biology Laboratory, Heidelberg, Germany*

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We describe the use of random peptide sequences for the mapping of antigenic determinants. An oligonucleotide with a completely degenerate sequence of 17 or 23 nucleotides was inserted into a bacterial expression vector. This resulted in an expression library producing random hexa- or octapeptides attached to a β-galactosidase hybrid protein. Mimotopes, or antigenic sequences that mimic an epitope, were selected by immunoscreening of colonies with monoclonal antibodies, which were specific for antigenic sites on the spike protein of the coronavirus transmissible gastroenteritis virus. We report one mimotope for antigenic site II, eight for site III and one for site IV. The site III and site IV mimotopes were closely similar to the corresponding linear epitopes, localized previously in the amino acid sequence of the S protein. An alignment of the site II mimotope and the sequence of the S protein around Trp97, which is substituted in escape mutants, suggests that this mimotope mimics a conformational epitope located around residues 97–103. Applications of mimotopes to epitope mapping, serodiagnosis and vaccine development are discussed.

Key words: Epitope; Mimotope; Random sequence; Expression library

Introduction

There are several methods for studying the antigenicity of proteins, each able to reveal a different aspect of the antibody-binding site (Van Regenmortel, 1989). Linear or sequential epitopes can be localized using synthetic peptides (Van Regenmortel, 1989) or prokaryotic expression products (Stanley and Herz, 1987; Lenstra et al., 1990). In the case of viral surface proteins, residues of discontinuous epitopes may be identified by analysing escape mutants (Page et al., 1988).

An alternative approach has been proposed by Geysen et al. (1986): the construction of oligopeptides that mimic the native epitope on the surface of the antigen. These peptides, so-

Correspondence to: J.A. Lenstra, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, P.O. Box 80.165, 3508 TD Utrecht, Netherlands. Tel.: (30) 534992; Fax: (30) 540784.

[1] *Present address:* The Heart Research Institute, Sydney, Australia.

Abbreviations: mAb, monoclonal antibody; TGEV, transmissible gastroenteritis virus.

called mimotopes, can be found by a systematic ab initio approach without using the primary structure of the antigen. First, the binding of all possible (400) dipeptides to the mAb is tested with the mAb of choice, then the most antigenic dipeptides are selected to synthesize tripeptides, and so on. This has led to the construction of a hexapeptide that was recognized specifically by a mAb against foot-and-mouth-disease virus. However, this procedure was rather time-consuming, while it was not entirely clear how this mimotope was related to the actual antibody-binding site on the virus. A similar approach has been used by Houghten et al. (1991).

Recently, more efficient procedures to obtain antigenic peptides have been described (Cwirla et al., 1990; Devlin et al., 1990; Scott et al., 1990). This approach is based on the expression of random oligonucleotides on the surface of filamentous phages. Antigenic phages are subsequently selected using biotinylated antibodies and immobilized on a streptavidin coating layer.

In this paper, we have used a similar method to identify mimotopes that correspond to three viral epitopes. Short random sequences were expressed in a prokaryotic plasmid expression system. Mimotopes were then selected by filter immunoscreening of colonies with monoclonal antibodies against the spike protein of the porcine coronavirus TGEV (Correa et al., 1988). The epitopes recognized by these antibodies had been mapped previously using bacterial fusion proteins, PEPSCAN analysis and escape mutants (Correa et al., 1989; Posthumus et al., 1990a,b; Gebauer et al., 1991). We obtained ten mimotopes specific for the viral epitopes, one of which was conformational. The agreement between the mimotopes and the antigenic TGEV sequences demonstrated that random-sequence expression libraries may be used for the mapping of antigenic determinants.

Materials and methods

Monoclonal antibodies

MAbs 1D.B12, 1B.H11, 6A.A6 and 5B.H1 have been described previously (Correa et al., 1988, 1989). MAbs 57.9, 57.24, 57.51 and 57.99 were provided by Dr. Van Nieuwstadt (Lelystad, Netherlands).

Plasmids

The expression vector pCL627 is a derivative of pEX627 (Stanley et al., 1987) with an *Eco*RI site just upstream of the P_R promotor, a *Bam*HI between the *cro* and *lacZ* gene fragments, but without the *Hin*dIII site outside the polylinker region. As in pEX627, nucleotides 627–3195 of pEX2 (Stanley et al., 1984; EMBL data bank accession code AREX2) have been deleted.

Construction of random-sequence expression libraries

100 pmol of the oligonucleotide 5′-CGGGT $(N)_m$GAAGCTTC (N = A, C, G or T, m = 17 or 23 for the hexapeptide and octapeptide libraries, respectively) were dissolved in 10 μl high-salt restriction enzyme buffer, heated at 70°C for 3 min and then cooled to room temperature to allow annealing of the 8 bp self-complementary 3′ end. Extension by Klenow polymerase (5 U, 1 mM of all 4 dNTPs, 10 min at 20°C followed by 60 min at 15°C) resulted in a double stranded random sequence dimer with a central *Hin*dIII site (AAGCTT). This dimer was purified on a Qiagen tip, precipitated with ethanol and ligated into the blunt *Sma*I site of pCL627 using 10 U T4 ligase in 20 μl at 20°C overnight. After 3 min at 70°C and adjusting the salt concentration, the *Hin*dIII sites in the dimer and at the polylinker were cut with 5 U of enzyme. The mixture was purified by phenol/chloroform extraction and ethanol precipitation and spun through a 1 ml bed of Sephacryl S400 in 1 mM Tris/HCl (pH 8.0), 0.1 mM EDTA, to remove the excised fragments. The DNA was recircularized via the *Hin*dIII sites in 30 μl with 5 U T4 ligase at 15°C for 1 h. Vector molecules without inserts were removed by cutting with 8 U *Sal*I for 30 min, after which the DNA was extracted with phenol/chloroform and precipitated with ethanol. Transformation of *E. coli* pop 2136 (Stanley et al., 1987) by the Hanahan procedure or, after microdialysis on Millipore 0.025 μm filters, by electroporation, yielded libraries of more than 10^7 clones.

Immunoscreening, gene expression, and Western blotting

After growing colonies on plates of 15 × 25 cm (about 10,000/plate) and transfer to nitrocelulose filters (S&S BA85) immunoscreening was carried out essentially as described previously (Stanley et al., 1983; Lenstra et al., 1989). The colonies were solubilized by saturating the filters with 5% SDS and baking for 5 min at 95°C. SDS was removed by electrophoresis in a Western blot apparatus filled with 192 mM glycine, 25 mM Tris, 20% (v/v) methanol (pH 8.3), with the solubilized (glassy) colonies facing the negative electrode and with a voltage of 50 V for 30 min. Next, the filters were incubated as in a normal Western blotting procedure with 0.5% gelatin, 0.1% Triton X-100 in PBS as dilution and wash buffer, a mAb dilution of 1/1000, alkaline phosphatase-conjugated secondary antibodies, and nitroblue tetrazolium and 5-bromo-4-dichloro-3-indolyl phosphate as staining reagents. Colonies on the masterplate corresponding to spots on the filter were localized after staining the filter for a few seconds in 0.6% Ponceau S in 3% trichloroacetic acid followed by destaining in tap water. Positive clones were purified by replating and immunoscreening with the antibody saved after the primary screening. Induction of gene expression, isolation of expression products and Western blotting were performed as described previously (Lenstra et al., 1989).

Sequencing of inserts

DNA of positive pCL627 recombinants was isolated by alkaline lysis (Maniatis et al., 1982) and used as a template for dideoxysequencing with [α-^{32}P]dATP and T7 DNA polymerase (Pharmacia) essentially as described by the manufacturer. The oligonucleotides 5′-GCCAGACGC-GAATTATTTTTG-3′ (nucleotides 590–610 from pEX2) and 5′-CTAGAGCCGCATCGATCCGG-ATC-3′ (complementary to 3243–3264 from pEX2) were used as primers. The first nucleotide incorporated during the extension of these primers is an adenosine residue; labelling with only [α-^{32}P]dATP (without any unlabelled

TABLE I

MONOCLONAL ANTIBODIES AGAINST THE SPIKE PROTEIN OF TGEV

Site [a]	mAb	Linear [b]	Neutralizing activity	Epitope [c]	Method of localization [d]	Reference
I (A)	1A.F10	+	+	543	mar	Gebauer, 1991
				537–547	PEPSCAN	Gebauer, 1991
	1D.E7	–	+	586, 591	mar	Gebauer, 1991
	1G.A7	–	+	538, 586	mar	Gebauer, 1991
	6A.C3	–	+	543	mar	Gebauer, 1991
II (B)	1B.H11	+	–	1–325	Expression	Correa, 1989
				97, 144	manb	Gebauer, 1991
	1D.B12	–	–	97	manb	Gebauer, 1991
III (C)	5B.H1	+	–	49–52, 165–168	PEPSCAN	Gebauer, 1991
	6A.A6	+	–	49–52, 165–168	PEPSCAN	Gebauer, 1991
IV (D)	57.9	+	+	380–387	PEPSCAN	Posthumus, 1990b
	57.24	+	+	380–387	PEPSCAN	Posthumus, 1990b
	57.51	+	–	379–386	PEPSCAN	Posthumus, 1990b
	57.99	+	+	380–387	PEPSCAN	Posthumus, 1990b

[a] Antigenic sites indicated by Roman numerals (Posthumus et al., 1990a, b) or letters (Correa et al., 1988, 1989; Gebauer et al., 1991).

[b] An epitope of an mAb is considered to be linear (as opposed to conformational) if the antigenicity does not depend on the native conformation of the antigen. The most practical criterion for linearity is the reactivity of the mAb on a Western blot. This correlates well with the ability to bind synthetic peptides or prokaryotic expression products that contain a segment of the sequence of the antigen (Lenstra et al., 1990).

[c] Numbers refer to the amino acid sequence of the TGEV spike protein precursor (Jacobs et al., 1987).

[d] Expression, localization by expression of gene segments in the prokaryotic pEX vector; manb, localization by sequencing mAb-nonbinding mutants; mar, localization by sequencing mAb-resistant mutants.

dNTPs) prior to the termination reactions permitted the reading of the sequence starting at 8–10 nucleotides from the primers.

Results

Expression and selection of random sequences

Fig. 1 shows the insertion of the random 17-nucleotide sequence between the *Sma*I and *Hin*dIII sites of pCL627. These sites are situated at the 3′ end of a truncated *cro-lacZ* fusion gene. Gene expression leads to a random hexapeptide sequence near the C terminus of the hybrid *cro*-β-galactosidase protein. In the same way, random octapeptides were produced by insertion of a 23-nucleotide random sequence. Presumably, the proline and glycine residues encoded by the *Sma*I site favour a surface exposure of the peptide.

Nucleotide sequencing of 15 clones that were randomly selected from the hexapeptide library showed that 11 clones carried one oligonucleotide insert, two carried two oligonucleotides and two had smaller inserts. As shown below, most of the clones that were selected by monoclonal antibodies carried shorter inserts, while none of the clones conformed exactly to the structure shown in Fig. 1. Presumably, these deviations resulted from errors during the oligonucleotide synthesis or from anomalous annealing events during the library construction. Furthermore, the binding requirements of a mAb may select clones with specific deviations, e.g., clones without intact *Sma*I sites (see below). As shown in Fig. 1, stop codons in all three reading frames end the translation irrespective of the length of the insert. To date, all clones isolated from the octapeptide bank contained single inserts (unpublished results).

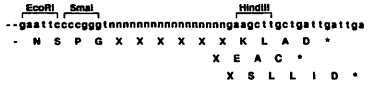

Fig. 1. Design of the random hexapeptide expression library. The *Sma*I and *Hin*dIII sites of plasmid pCL627 were used to insert a double-stranded oligonucleotide with a degenerate sequence as indicated.

TABLE II

SCREENING OF THE RANDOM-SEQUENCE EXPRESSION LIBRARIES

mAb used for screening [a]	Site	Number of colonies tested	Number of positives	mAbs recognizing positives
(A) Hexapeptide library				
1G.A7, 1D.E7, 6A.C3, 1A.F10	I	80,000	–	–
1D.B12, 1B.H11	II	240,000	1	1D.B12
6A.A6	III	160,000	7	6A.A6
				5B.H1
57.9	IV	160,000	1	57.9
				57.24
				57.99
(B) Octapeptide library				
6A.A6	III	300,000	5	6A.A6 [b]

[a] The screening with site I- and site II-specific mAbs was performed with pooled mAbs; after the purification of the positive clone for site II, its specificity for the individual mAbs was determined by Western blotting. [b] Other mAbs not tested.

We carried out immunoscreening of the hexapeptide library with eight different mAbs specific for antigenic sites on the spike protein of TGEV (Table I). In addition, a site III mAb was used to select clones from the octapeptide library. Most epitopes recognized by the site I and site II specific mAbs are conformational (Correa et al., 1988) and have been characterized by analyzing nonbinding mutants (Gebauer et al., 1991). The site III and site IV epitopes are linear and have been localized by prokaryotic expression of TGEV gene fragments (Correa et al., 1989).

Three of the mAbs selected antigenic clones (Table II). The recognition was quite specific as shown by the typical immunoscreening pattern (not shown; see Fig. 4 of Stanley et al., 1984), i.e., an unmistakable staining of positive colonies above the low background signals of other colonies. After purification of the positive clones, the *cro-lacZ* hybrid proteins from these clones were analysed by Western blotting (Fig. 2). Again, the expression products with apparent molecular weights around 30,000 were recognized unam-

biguously by the mAbs used to select the clones. Similar results were obtained with antigenic octapeptide clones (not shown). In order to correlate the sequences of the antigenic clones with information on the respective mimotopes, the nucleotide sequences of the inserts were determined and translated into protein sequences (Fig. 3).

Site III

PEPSCAN analysis showed that site III (or C) is specified by a short motif that occurs twice in the S sequence (Posthumus et al., 1990a; Gebauer et al., 1991). The site III specific mAbs cross-reacted with a hitherto unidentified component of sera from different species (unpublished results).

Screening of the random-hexapeptide library with the site III specific mAb 6A.A6 yielded seven clones. Interestingly, on Westerns blots hybrid proteins of these clones were antigenic with both 6A.A6 and another site III specific mAb (5B.H1, see Fig. 2). Nucleotide sequencing showed that clone #5, which produced a protein with a relatively low mobility (Fig. 2), contained a plasmid with more than one binding site for the sequencing primers; this clone was not analysed further. Two antigenic clones from the octapeptide library were also purified; sequence analysis indicated that these were identical clones (mimotope #10).

Aligning the peptide sequences encoded by the nucleotide sequences of the inserts with the antigenic motifs from the S sequence (residues 48–52 and 164–169, respectively) revealed a clear agreement between the mimotopes and the two epitopes (Fig. 3). In fact, the mimotope of clone #6 is for six consecutive residues identical to one of the two viral epitopes.

The information on the sequences that are recognized by the site III mAbs may prove useful for the identification of the cross-reacting serum components.

Site IV

Neutralizing mAbs specific for site IV (or D) recognize a pEX expression product carrying the TGEV S sequence 326–558 (Correa et al., 1989). By PEPSCAN analysis, the epitope was further localized within the linear sequence 380–387 (Posthumus et al., 1990b). Systematic and consecutive replacement within the sequence 380–387 showed that substitutions of the residues 382, 383

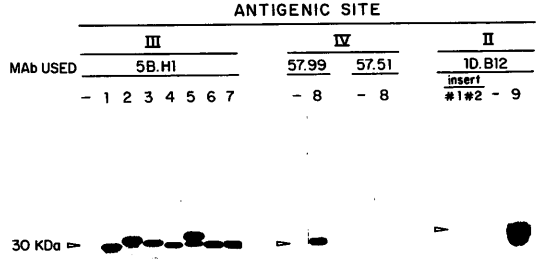

Fig. 2. Antigenicity of hybrid proteins from clones selected from the hexapeptide library by immunoscreening. The numbering of clones above the lanes corresponds with the numbering in Table I and Figure 3. –, negative clone from the expression library; 1–7, hexapeptide clones #1 to #7, respectively. The patterns with mAbs 6A.A6 and 5B.H1 were identical. 8, clone #8. The patterns with mAbs 57.9, 57.24 (not shown) and 57.99 were essentially identical. Insert #1, pEX clone with insert coding for TGEV spike residues 1–378 (Correa et al., 1989); insert #2, insert coding for residues 326–558; 9, clone #9 selected with mAb 1D.B12. The broad band of clone #9 was caused by the fast and intense colour development with mAb 1D.B12.

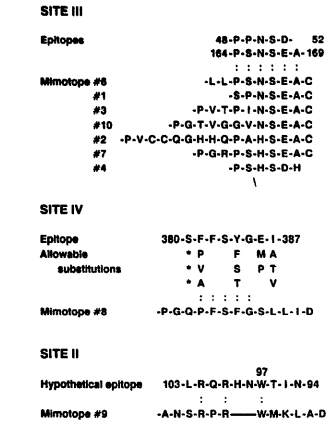

Fig. 3. Alignment of epitopes and mimotopes. The epitopes from sites III and IV are based on PEPSCAN analysis (Posthumus et al., 1990a,b; Gebauer et al., 1991); the hypothetical epitope of site II is based on the analysis of escape mutants (Gebauer et al., 1991). : indicates a perfect match of the mimotope sequence with the sequence or the epitope or a match with one of its allowable substitution variants (Posthumus et al., 1990a). * indicates that any substitution is tolerated. Residues of the site II mimotope that are encoded by the vector sequence are placed between parentheses.

and 385 destroyed the antigenicity, while a limited number of substitutions were tolerated at positions 381, 384, 386 and 387. The nonneutralizing site IV specific mAbs 57.51 and 1D.G3 recognized overlapping sequences in the same region (Posthumus et al., 1990b).

Screening with the neutralizing mAb 57.9 yielded one mimotope. This mimotope was also recognized by the neutralizing mAbs 57.24 and 57.99, but not by the nonneutralizing mAb 57.51 (Fig. 2). Alignment of this mimotope with the 380–387 sequence (Fig. 3) again shows a close resemblance: the mimotope contains not only the three essential residues Phe382, Ser383 and Gly385, but also conforms to the allowable substitutions at positions 381 and 384.

Site II

mAbs directed against site II (or B) are capable of discriminating between TGEV and the closely relatcd porcine respiratory coronavirus (PRCV) (Sánchez et al., 1990). Correa et al. (1989) localized this site within residues 1–325. The site II specific mAb 1D.B12 probably recognizes a conformational epitope, since it does not bind to SDS-treated virus (Correa et al., 1988), prokaryotic expression products of the 5′ end of the S gene (Correa et al., 1989; see Fig. 2 for inserts #1 and #2) or PEPSCAN nonapeptides (not shown). However, by analyzing three 1D.B12 nonbinding mutants (Gebauer et al., 1991), it was found that Trp97 of the wild-type sequence was critical for the binding of the mAb.

The same antibody selected a mimotope sequence from the random expression library. The relatively rapid intense staining of the expression product (Fig. 3) suggests that the mimotope is highly antigenic. Strikingly, the sequence of the mimotope (-Arg-Pro-Arg-Trp-Met-Lys-Leu-Ala-Asp) contains not only a tryptophan, but also a basic motif (Arg-Pro-Arg) that is very similar to the Arg-Gln-Arg sequence at positions 100–102. We hypothesize that residues 97, 100 and 102 of S protein are part of site II and that the spacing of these residues in the mimotope sequence mimics the configuration of the authentic 1D.B12 epitope.

The proposed location of site II, which is not conserved in TGEV strains (Delmas et al., 1986; Sánchez et al., 1990) indicates that the N terminal region of the S protein is relatively variable. This is in agreement with a comparison of the sequences of TGEV and the closely related feline infectious peritonitis virus (Jacobs et al., 1987).

Discussion

Mimotopes and the mapping of epitopes

Recently, random-expression libraries have been used to identify sequences recognized by streptavidin (Devlin et al., 1990) or by mAbs raised against a synthetic peptide (Scott et al., 1990) or against a peptide hormone (Cwirla et al., 1990). A related approach is the use of a random-sequence synthetic peptide library (Lam

et al., 1991). In this study, we used mAbs specific for a more complex antigen, the spike protein of the coronavirus transmissible gastroenteritis virus. The antigenic structure of this protein has been investigated previously with PEPSCAN peptides, prokaryotic expression products and/or escape mutants (Table I).

We wished to answer two questions: (1) Can we identify mimotopes by immunoscreening of a bacterial random-sequence expression library with antiviral mAbs? (2) If so, are these mimotopes useful for the mapping of the corresponding epitopes on the surface of the virus?

We have indeed demonstrated that sequences which mimic a viral epitope can be identified by immunoscreening of a hexa- or octapeptide library. Not surprisingly the number of antigenic clones in the library depended on the antibody used for immunoscreening. However, the figures in Table II are probably not the true frequencies of antigenic hexapeptides, since several clones deviate from the ideal structure shown in Fig. 1. Moreover, the proline (P) on the N terminal side and on E-A at the C terminal side of the site III mimotopes are encoded by the polylinker of pEX. This probably explains the relatively high frequency of random hexapeptides selected by the site III specific mAb. In the octamer library the selection of colonies by mAb 6A.A6 was less frequent.

We have also demonstrated that mimotopes are able to provide clues on the location of epitopes. Scanning the 1447 residues of the TGEV spike protein with a consensus sequence of the site III mimotopes, G/P/S-X-N/H-S-E/D, produces matches at positions 48-PPNSD-52, 164-PSNSE-168 and 754-SINSE-758. In fact, this faithfully reproduces the PEPSCAN pattern (Gebauer et al., 1991), although there were quantitative differences in antigenicity (48–52 > 164–168 ⋙ 754–758; this latter peak was observed only at high concentrations of antibody). In addition, the mimotopes provide information on substitutions that are allowed within the antigenic sequence.

The mimotope of the conformational site II can be aligned tentatively with the antigenic sequence localized by analysing escape mutants. This suggests that mimotopes can also be useful for the localization of conformational epitopes.

Our data indicate that the present expression library is most successful if only a few residues in close proximity are essential for antigenicity. Consequently, a strategy to obtain mimotopes that simulate more complex epitopes may be based on (a) the screening of more recombinants and (b) the use of longer degenerate sequences. Immunoscreening on filters permits the simultaneous test of up to a million colonies, depending on the availability of the antibody. With phage libraries even more transformants can be screened (Cwirla et al., 1990; Devlin et al., 1990; Scott et al., 1990).

The libraries of Cwirla et al. (1990) and Scott et al. (1990) contained, in common with the first pEX library used in this study, random peptides of six residues. Preliminary results with the pEX octapeptide library (Siddle and Stanley, unpublished) indicated a higher frequency of positive clones and a less frequent selection of aberrant structures, suggesting that most epitopes span more than six residues. In order to cover all possibilities, an octapeptide library should contain 20^8 ($= 2.56 \times 10^{10}$) different clones. However, the required number of clones is reduced considerably by the allowable substitutions within the antigenic sequence. Furthermore, residues essential for antigenicity could be identified by sequencing a number of weakly antigenic octapeptide clones (Siddle and Stanley, unpublished). Longer peptides, such as the pentadodecapeptides used by Devlin et al. (1990) would not only be useful for extended mimotopes, but also would reduce further the number of clones required for shorter mimotopes.

We conclude that screening of random-sequence expression libraries (Cwirla et al., 1990; Devlin et al., 1990; Scott et al., 1990; this work) is a general and efficient approach to the mapping of B cell epitopes. In principle, it obviates the synthesis of peptides and the cloning of gene fragments in an expression plasmid. Several mAbs can be combined during the primary screen and can be tested separately at a later stage. Likewise, positives selected by polyclonal antisera can be grouped by the immune-selection procedure

(Stanley et al., 1987). Furthermore, it may be of fundamental interest that this method provides an elegant approach to measuring the selectivity of a given antibody.

Other applications of mimotopes

Sequences that bind specifically to antibodies may have several useful applications, e.g., as peptide ligands for affinity purification or as specific drugs directed against antibodies involved in pathological processes.

From the outset it was suggested that mimotopes may also be used to formulate peptide immunogens for vaccine development (Geysen et al., 1986). Scientifically challenging as this may be there are a few caveats. Firstly, one should discriminate between structural and antigenic equivalence. Mimotopes are constructed or selected by satisfying the binding requirements of one particular antibody. Consequently, a mimotope may be a reconstruction of the antigenic surface that is biased by only one antibody rather than a realistic representation. In fact, we have observed that the mimotopes of sites II and IV from TGEV are not recognized by mAbs specific for other epitopes within the same site.

Secondly, if none of the antibodies in the repertoire of the infected animal has the same cross-reaction with the mimotope as the antibody used for the screening, this mimotope would be of no use at all for either serodiagnosis or vaccination. Thirdly, antigenic equivalence (recognition by the same antibody or antibodies) does not necessarily imply immunogenic equivalence (induction of the same antibodies). Posthumus et al. (1991) showed that the presentation of a peptide derived from the TGEV site IV had a drastic effect on the induction of antibodies and their cross-reaction with the TGEV spike protein.

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