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Identification of antigenic regions on VP2 of African horsesickness virus serotype 3 by using phage-displayed epitope libraries

Louise Bentley,[1,2] Jeanni Fehrsen,[1] Frances Jordaan,[1] Henk Huismans[2] and Dion H. du Plessis[1]

[1] Immunology Division, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort 0110, Republic of South Africa

[2] Department of Genetics, University of Pretoria, Pretoria, Republic of South Africa

VP2 is an outer capsid protein of African horsesickness virus (AHSV) and is recognized by serotype-discriminatory neutralizing antibodies. With the objective of locating its antigenic regions, a filamentous phage library was constructed that displayed peptides derived from the fragmentation of a cDNA copy of the gene encoding VP2. Peptides ranging in size from approximately 30 to 100 amino acids were fused with pIII, the attachment protein of the display vector, fUSE2. To ensure maximum diversity, the final library consisted of three sub-libraries. The first utilized enzymatically fragmented DNA encoding only the VP2 gene, the second included plasmid sequences, while the third included a PCR step designed to allow different peptide-encoding sequences to recombine before ligation into the vector. The resulting composite library was subjected to immunoaffinity selection with AHSV-specific polyclonal chicken IgY, polyclonal horse immunoglobulins and a monoclonal antibody (MAb) known to neutralize AHSV. Antigenic peptides were located by sequencing the DNA of phages bound by the antibodies. Most antigenic determinants capable of being mapped by this method were located in the N-terminal half of VP2. Important binding areas were mapped with high resolution by identifying the minimum overlapping areas of the selected peptides. The MAb was also used to screen a random 17-mer epitope library. Sequences that may be part of a discontinuous neutralization epitope were identified. The amino acid sequences of the antigenic regions on VP2 of serotype 3 were compared with corresponding regions on three other serotypes, revealing regions with the potential to discriminate AHSV serotypes serologically.

Introduction

African horsesickness virus (AHSV) causes a severe disease that often kills unvaccinated horses. It is a member of the viral genus *Orbivirus*, in the family *Reoviridae*. So far, at least nine serotypes have been defined by cross-neutralization with immune serum (Howell, 1962). The virion is structurally similar to bluetongue virus (BTV), the prototype of the genus, with each particle consisting of 10 double-stranded RNA segments enclosed within a capsid consisting of four major and three minor structural proteins (Huismans & Van Dijk, 1990). Of the major proteins, VP3 and VP7 comprise the core particle, while VP2 and VP5 make up the outer capsid. Three minor proteins (VP1, VP4 and VP6) are located within the core. In addition, three non-structural proteins (NS1, NS2 and NS3) are found in cells harbouring replicating virus (Roy, 1992).

Sequence comparisons show that, of the four major structural proteins, VP2 has the greatest variability between serotypes (Iwata *et al.*, 1992; Williams *et al.*, 1998). Moreover, together with VP5, it is one of two proteins recognized by virus-neutralizing antibodies (Burrage *et al.*, 1993; Vreede & Huismans, 1994; Martínez-Torrecuadrada & Casal, 1995; Martínez-Torrecuadrada *et al.*, 1999). Expression in *E. coli* of restriction enzyme fragments has broadly defined a neutralization domain located between amino acid residues 253 and 413 on VP2 of AHSV serotype 4 (Martínez-Torrecuadrada & Casal, 1995). While immunogenic regions capable of eliciting neutralizing antibodies have been mapped to a resolution of 34 residues with this serotype, very little is known regarding the sites on VP2 of any serotype recognized by the antibodies in an immune serum. Such information is

Author for correspondence: Dion du Plessis.
Fax +27 12 5299431. e-mail dion@moon.ovi.ac.za

0001-6771

likely to be of paramount importance in developing new immunodiagnostic approaches. For example, epidemiological studies and disease surveillance often require a knowledge of which serotype occurs in a particular area. Even if no virus can be isolated, this can be done by plaque-reduction assays with antiserum from infected or carrier animals (Barnard, 1993). For rapid serotyping, however, an ELISA that can distinguish antibodies to the different serotypes would have distinct advantages. Accordingly, it would be extremely useful to find out whether VP2 has any highly antigenic regions that can be mimicked by linear peptides and, at the same time, correlate with regions of significant inter-serotype sequence variability. A knowledge of where antibodies bind to VP2 could also contribute towards further characterization of neutralization domains and, in the absence of X-ray diffraction data, can give some indication of how the polypeptide is folded by identifying accessible amino acids. Computer programs can sometimes predict epitopes (Van Regenmortel & de Marcillac, 1988), but to locate the antigenic regions by experimental means probably remains a more reliable approach.

Filamentous phage display (Smith, 1985) offers a relatively direct and highly selective approach to the fine-mapping of epitopes on viral and other proteins. A collection of random fusion peptides encoded either by degenerate oligonucleotides (Scott & Smith, 1990; Devlin *et al.*, 1990; Cwirla *et al.*, 1990; Kay *et al.*, 1993) or fragments of a target gene (Wang *et al.*, 1995; Petersen *et al.*, 1995) is expressed as part of the attachment protein pIII (Smith, 1985; Parmley & Smith, 1988) or the major capsid protein pVIII (Felici *et al.*, 1991; Ilyichev *et al.*, 1992). The foreign peptides are accessible on the phage surface. Consequently, phages displaying antigenic sequences can be selected specifically from a library by binding to immobilized antibodies. The peptide is identified by sequencing the phage DNA. With the orbiviruses, phage display techniques have so far been used to map antigenic determinants on VP7 (Du Plessis *et al.*, 1994), VP5 (Wang *et al.*, 1995) and NS1 (Du Plessis *et al.*, 1995) of BTV.

To locate epitopes on VP2 of AHSV, a filamentous phage library (Lib-VP2) displaying short peptides derived from fragments of the VP2 gene of AHSV serotype 3 (AHSV-3) was constructed and screened with polyclonal antisera and a neutralizing monoclonal antibody (MAb). By using this approach, several continuous epitopes located in the N-terminal half of the polypeptide could be identified. Important binding areas were mapped with high resolution by identifying the minimum overlapping areas of the selected peptides. In addition, by using a MAb and a random 17-mer epitope library (Bonnycastle *et al.*, 1996), residues possibly involved in the formation of a discontinuous neutralizing epitope could be identified. Finally, a comparison of the antigenic regions identified by phage display with corresponding regions on three other serotypes revealed at least two regions with the potential to discriminate serologically between AHSV serotypes.

Methods

■ **Plasmids, bacteria and random epitope library.** *E. coli* strains MC1061 and K91 and the phage display vector fUSE2 (Parmley & Smith, 1988) were kindly provided by G. P. Smith (University of Missouri, USA). The XCX_{15} epitope library was a generous gift from Lori Bonnycastle (Simon Fraser University, Burnaby, BC, Canada). Plasmid pBS-VP2 carried the full-length gene of VP2 of AHSV-3 (Vreede & Huismans, 1994).

■ **Antigens and antibodies.** VP2 of AHSV-3 (Vreede & Huismans, 1994) truncated by 305 C-terminal residues (VP2-tr) was constructed by subcloning into the pFastBacHTc vector and expressed by recombinant baculoviruses in *Spodoptera frugiperda* (Sf9) cells (Life Technologies). AHSV-3-specific neutralizing chicken IgY was obtained by PEG precipitation (Polson *et al.*, 1985) from the yolk of an egg laid by a Leghorn hen immunized with density-gradient-purified AHSV (Huismans *et al.*, 1987). Protein-specific antibodies were isolated from electroblotted VP2-tr (Du Plessis *et al.*, 1995) and their specificity for VP2 was reconfirmed by immunoblotting. AHSV-specific IgY was immunoaffinity-purified on whole virus immobilized on a 4% cross-linked beaded agarose support according to the manufacturer's instructions (Pierce). Anti-AHSV equine antibodies were purified by ammonium sulphate precipitation and DEAE ion-exchange chromatography (Clark & Adams, 1977) from antiserum (a gift of C. Vroon, Onderstepoort Veterinary Institute) obtained from a horse infected with cell culture-attenuated AHSV-3. The neutralizing MAb 2F2 (Van Wyngaardt *et al.*, 1992) was purified from ascitic fluid as above.

■ **Fragmented-gene libraries.** Libraries displaying AHSV peptides were constructed according to methods described by Wang *et al.* (1995) and Du Plessis & Jordaan (1996). In essence, the double-stranded 'replicative form' (RF) of the display vector fUSE2 DNA and pBS-VP2 plasmid DNA were prepared by alkaline lysis and CsCl–ethidium bromide gradient centrifugation (Sambrook *et al.*, 1989). Vector DNA was digested with *Bgl*II, dephosphorylated and recovered after agarose gel electrophoresis (Glassmilk, BIO 101). Random fragmentation of target DNA encoding VP2 (pBS-VP2) with DNase I was used to produce fragments of between 50 and 300 bp. Library 1 utilized the VP2 gene excised from the vector as target, while the entire plasmid was used for Library 2. For the linker-ligated PCR library (Library 3), approximately 200 ng blunt-ended fragments was incubated in ligation buffer in the presence of 1 U T4 DNA ligase (Boehringer) before adding 250 pmol phosphorylated *Bam*HI linkers. The linker-ligated DNA was then amplified by PCR (Nagesha *et al.*, 1996).

Modified DNA fragments of 100–300 bp were separated electrophoretically on 5% polyacrylamide gels. Recovered fragments were ligated into approximately 400 ng linear dephosphorylated fUSE2 DNA. One-fifth of a 10 µl ligation was electroporated into electrocompetent MC1061 cells. Transformed clones were selected on LB agar containing 40 µg/ml tetracycline (Tet). Resulting libraries were harvested by scraping the colonies into Tris-buffered saline (TBS), removing cells by centrifugation and precipitating phages twice with 0·15 vols 16·7% PEG, 3·3 M NaCl (PEG/NaCl). Each library was titrated as transducing units (TU) (Smith & Scott, 1993). Colony PCR (Wang *et al.*, 1995) was used to screen for bacterial clones that contained fragments of DNA inserted at the *Bgl*II site of the vector by using primers III-5 (5′ GGTTGGTGC-CTTCGTAGT 3′) and III-3 (5′ CCATGTACCGTAACACTG 3′). The three different libraries were pooled, resulting in a combined library designated Lib-VP2.

■ **Affinity selection by panning.** Panning of the phage libraries was done in ELISA plate wells by using antibody preparations adsorbed

directly to the plastic surface (Wang *et al.*, 1995). Any phage-binding antibodies were blocked by adding 50 µl UV-killed f1 phage particles suspended in 1% BSA in TBS containing 0·05% Tween 20 prior to panning. After washing, approximately 4×10^{10} TU of the phage library suspended in 200 µl of the same buffer was used in the first round of selection.

■ **Sequencing of selected inserts.** Single-stranded DNA of phage clones selected by panning was prepared by standard methods (Sambrook *et al.*, 1989). Cycle sequencing with dye-labelled terminators was with the primer 5′ CCCTCATAGTTAGCGTAACG 3′ and the Big Dye Ready reaction mix (PE Applied Biosystems). PCR products were analysed with an ABI PRISM 310 Genetic Analyser. DNA from the XCX_{15} library was sequenced manually (Sequenase kit) by using the f88.4 sequencing primers (Bonnycastle *et al.*, 1996).

■ **ELISA.** Phages were multiplied in *E. coli* K91 in LB broth containing 40 µg/ml Tet and isolated from the culture supernatant by precipitation with 0·15 vols PEG/NaCl. Phages were diluted to 20 µg protein/ml in TBS and 50 µl aliquots were used to coat microtitre plate wells (Corning, Easywash) overnight at 4 °C. ELISAs were done in triplicate as described previously (Du Plessis *et al.*, 1995). In some experiments, antibodies were cross-absorbed by incubating at least 5×10^{10} phage particles per µg antibody at 37 °C for 60 min in PBS containing 0·5% Tween 20 and 1% (w/v) non-fat milk powder prior to ELISA.

Results

Construction and characterization of a fragmented-gene epitope library

Three 'precursor' filamentous phage libraries were constructed, each displaying short peptides derived from the VP2 gene of AHSV-3. The aim was to maximize the diversity of peptide sequences available for binding by the AHSV antibodies. The first (Library 1) used fragments of the VP2 gene after its excision from the cloning vector. Accordingly, any meaningful DNA fragment at the extremity of the target gene, or one that ends with a *Bam*HI restriction site, will be expressed as a fusion peptide with its terminal residues invariably adjacent to the N terminus of the phage pIII attachment protein. If, however, the entire plasmid, rather than just the insert, is randomly digested, such termini could potentially be incorporated into some DNA fragments that also include nucleotides from the plasmid. This would have the effect of randomizing the context in which terminal peptides, or those derived from DNA with *Bam*HI sites, are fused to the phage. For this reason, Library 2 was constructed using DNA fragments of the entire plasmid that contained the target gene. Moreover, these fragments were modified with *Bgl*II, rather than *Bam*HI linkers. Finally, Library 3 was constructed in an attempt to juxtapose different DNA fragments randomly and thereby conceivably recreate discontinuous antigenic determinants when expressed. After blunt-ending, the fragments were allowed to ligate with each other before being amplified by PCR and ligated into the display vector (Nagesha *et al.*, 1996).

PCR amplification was used to determine the size and frequency of the VP2-derived inserts. With Library 1, 51·5% of the colonies had inserts, with a mean size of 130 bp. Assuming that only 1/18 of the c.f.u. in a primary library can produce an infective phage particle displaying an authentic sequence (Parmley & Smith, 1988; Wang *et al.*, 1995), it required 2012 c.f.u. with inserts to obtain a 99% probability of representing the entire VP2 gene (Clarke & Carbon, 1976). Similarly, Library 2, with its mean insert size of 73 bp and inserts in 78% of the clones, required 7226 c.f.u. with inserts. The actual number of c.f.u. was $1{\cdot}3 \times 10^4$ for Library 1 and $3{\cdot}2 \times 10^5$ for Library 2. Each was therefore likely to be sufficiently large to represent VP2 in its entirety. In addition, DNA from several Library 3 clones was sequenced, both before and after affinity selection, but phages displaying anything other than a single contiguous VP2 sequence were rare and did not identify any novel epitopes after panning (not shown). The three precursor libraries were pooled by mixing $1{\cdot}12 \times 10^{10}$ TU from Library 1, $2{\cdot}8 \times 10^9$ TU from Library 2 and $1{\cdot}9 \times 10^{10}$ TU from Library 3, resulting in the composite display library, Lib-VP2.

Selection with antibodies immunoaffinity-purified on electroblotted VP2-tr

IgY antibodies directed against purified AHSV were raised in a chicken. To enrich for those most likely to recognize continuous epitopes, recombinant viral protein was separated by SDS–PAGE and transferred to a PVDF membrane for use as an immunoaffinity matrix. Since the 305 C-terminal residues of VP2 were not antigenic in immunoblotting (L. Bentley, unpublished), it was possible to use a baculovirus-expressed truncated version of VP2 (VP2-tr) in this step. Lib-VP2 was panned with eluted antibodies at a concentration of 10 µg/ml for two rounds and at 1 µg/ml for a third. A total of 25 phage clones with VP2-derived inserts in the correct orientation and reading frame were selected (Fig. 1). Comparison with the authentic VP2 sequence indicated that most encoded fusion peptides clustering in three regions located between amino acid residues 323 and 529 (Fig. 1*b–d*). Seven peptides overlapped in the region encompassing residues 324 and 365. These all contained the sequence IRRA (Fig. 1*b*). Similarly, peptides displayed by the clones in the other two clusters overlapped residues HKAEVKKFL (Fig. 1*c*) or QGTRTAA-IVET (Fig. 1*d*). In addition, two single clones, one displaying residues nearer the N terminus (228–282) and the other towards the C terminus, were also selected (Fig. 1*a*, *e*).

Affinity selection with IgY released from whole AHSV

In order to allow peptides selected with antibodies released from denatured VP2-tr to be compared with those directed against VP2 in its context as part of the AHSV capsid, virus-specific chicken IgY was immunoaffinity-purified on immobilized whole virus. Eluted antibodies were too dilute to measure spectrophotometrically and were therefore used undiluted for rounds 1 and 2 and diluted 1:10 for round 3. Fifteen sequenced clones contained meaningful inserts

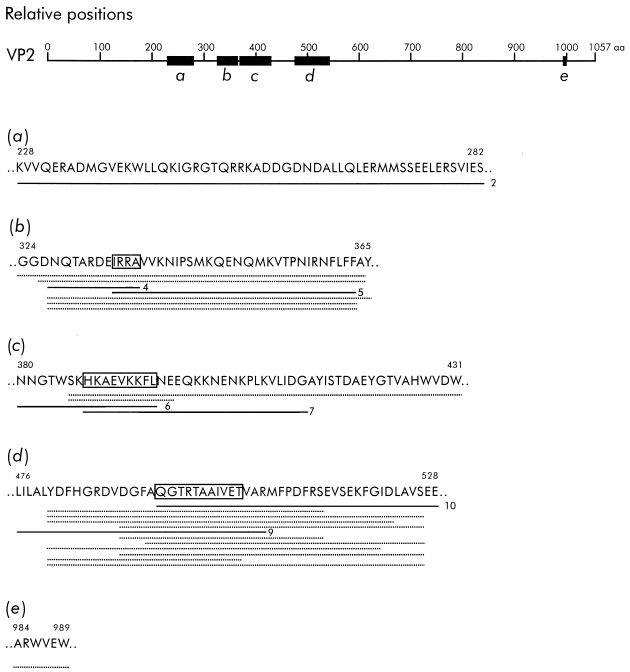

Fig. 1. Amino acid sequences of fusion peptides selected from Lib-VP2 by panning with electroblot-eluted IgY and their relative positions on VP2. Each dotted or solid line represents the peptide displayed by a selected phage clone. Boxed amino acid residues were common to all peptides identified in that region. Peptides represented by solid lines were selected for further characterization.

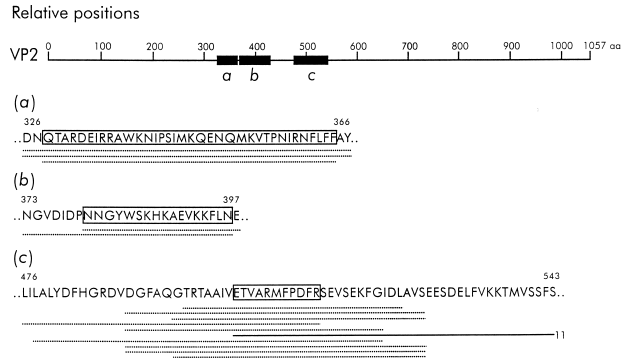

Fig. 2. Amino acid sequences of fusion peptides selected from Lib-VP2 by panning with virus-eluted IgY and their relative positions on VP2. Each dotted or solid line represents the peptide displayed by a selected phage clone. Boxed amino acid residues were common to all peptides identified in that region. Peptide 11 was selected for further characterization.

representing VP2 peptides (Fig. 2). Two regions between amino acids 325 and 398 (Fig. 2*a, b*) and another between 475 and 544 (Fig. 2*c*) were identified as antigenic. The overlapping four residues IRRA depicted in Fig. 1(*b*) are located within the overlap region shown in Fig. 2(*a*). Despite affinity purification on whole AHSV, these antibodies selected no peptide sequences nearer to the N terminus than amino acid residue 326. Moreover, none located nearer to the C terminus than residue 543 were selected, confirming that this part of VP2 is not highly antigenic. Overall, there was a marked similarity to

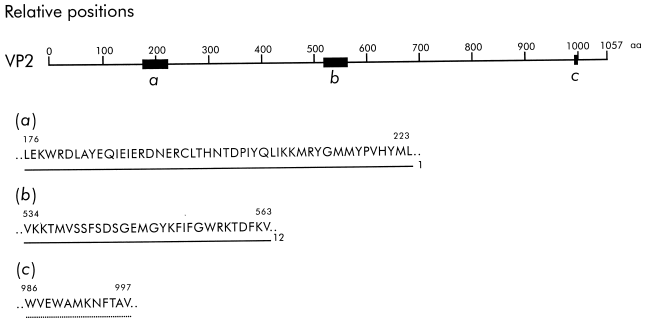

Fig. 3. Amino acid sequences of fusion peptides selected from Lib-VP2 by panning with MAb 2F2 and their relative positions on VP2. Peptides 1 and 12 were selected for further characterization.

Table 1. Sequences of fusion peptides displayed by phage clones isolated from the random XCX_{15} library and their immunoreactivity in ELISA

Peptide	Sequence and no. of clones	A_{450}
X1	TCPGEDCHT**MYP**FT**Y**RGY (11)	0·197
X2	SCPGEDCHT**MYP**FT**Y**KGY (14)	0·229
X3	TCPGEDCHS**MYP**FT**Y**RGY (1)	0·259
X4	RCQFSVWSWESLRCEFPY (2)	0·861
X5	RCHQNGVCMHRNVLLTTY (1)	0·149
X6	RCDLYPDGNLWGMPCPTY (1)	0·185
X7	NCNP**LEKW**CPTVEQTPWY (1)	0·780
X8	VCHAGELPFTFRGVPFPY (2)	0·185

the regions identified by the antibodies against denatured VP2. Exceptions were the single copy of peptide 2 (Fig. 1*a*) and the sequence ARWVEW (Fig. 1*e*).

Affinity selection of fusion peptides with MAb 2F2

The MAb 2F2 immunoprecipitates VP2 (and to a lesser extent VP7) of AHSV-3 and reduces the number and size of its plaques (Van Wyngaardt *et al.*, 1992). It was used to pan Lib-VP2 at a concentration of 10 μg/ml for two rounds and 1 μg/ml for one round. Sequencing the DNA of 12 phage clones revealed that only three had meaningful VP2 inserts, while the remainder contained either vector or linker sequences (Fig. 3). This high background suggested that the MAb had a low affinity for the linear fusion peptides in the library. In view of this, an attempt was made to ascertain whether any peptides mimicking its recognition sequence could be identified. Accordingly, MAb 2F2 was used to screen a phage library displaying a repertoire of random 17-residue peptides fused to pVIII (XCX_{15} library; Bonnycastle *et al.*, 1996). To permit the formation of disulphide-stabilized loops, this library has a fixed cysteine adjacent to the N-terminal amino acid of each peptide. Table 1 shows that the MAb identified eight different peptide sequences.

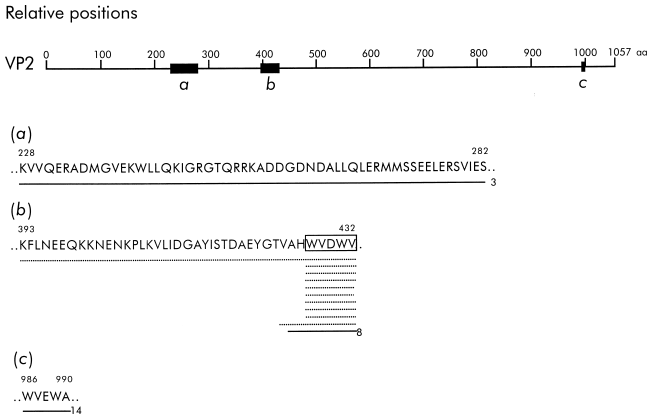

Fig. 4. Amino acid sequences of fusion peptides selected from Lib-VP2 by panning with horse anti-AHSV-3 IgG and their relative positions on VP2. Peptides 3, 8 and 14 represented by solid lines were selected for further characterization.

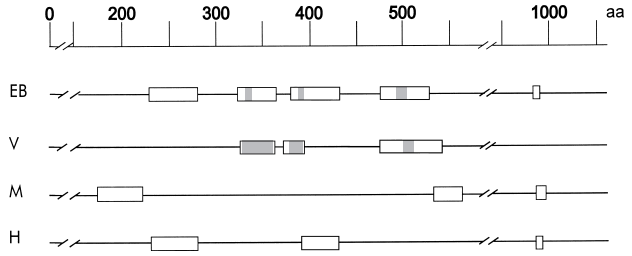

Fig. 5. Relative positions on the VP2 polypeptide of peptides selected from Lib-VP2 with various antibody preparations (EB, electroblot-eluted IgY; V, virus-eluted IgY; M, MAb 2F2; H, horse IgG). Shaded boxes represent residues common to all the peptides selected by a preparation of antibodies in a particular region, while open boxes show the entire region spanned by those peptides.

Comparison with VP2 revealed that the sequence LEKW (Table 1, peptide X7) was represented at positions 176–179 (Fig. 3*a*), while MYPFTY (Table 1, peptides X1, X2 and X3) was present at residues 216–221 of the viral protein.

Affinity selection with horse antibodies

AHSV does not normally replicate in chickens or mice. To permit epitopes mapped with IgY and the MAb to be compared with those recognized by a natural host's immune system during infection, immunoglobulins from an infected horse were used to pan Lib-VP2. An IgG concentration of 20 μg/ml was used for the first two rounds and 2 μg/ml for the final round. Sequencing revealed a peptide representing amino acids 228–282 (Fig. 4*a*; peptide 3). It was identical to peptide 2 (Fig. 1*a*), which was selected with IgY released from the electroblotted VP2-tr. A further 11 clones, eight of which were identical siblings, displayed peptides that included residues 393–432, with a minimal overlap (398–432) shifted relative to overlaps identified by chicken antibodies (Figs 4*b* and 5). A further potentially antigenic region near the C terminus (Fig. 4*c*, peptide 14) was also identified.

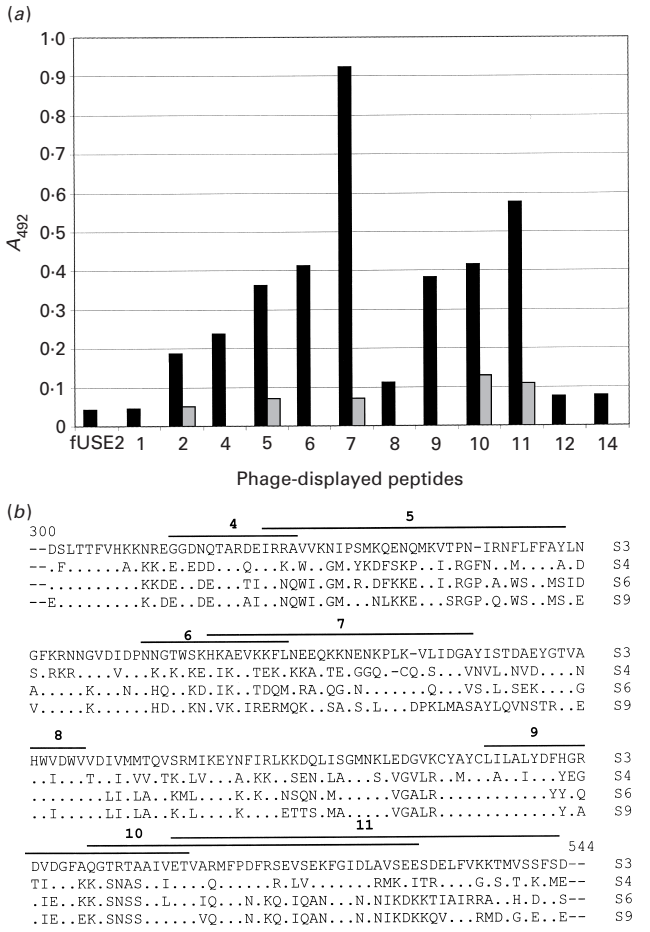

Fig. 6. (*a*) Reactivity with IgY of phage-displayed peptides in ELISA. Filled columns represent ELISA signals of peptides 1, 2, 4–12 and 14 with chicken anti-AHSV-3 cross-absorbed with non-fusion fUSE2 phages. Shaded columns represent signals after further cross-absorption with their homologous fusion phage-displayed peptides (not done for peptides 1, 12 or 14). (*b*) Sequence alignment of AHSV VP2 amino acid residues 300–544 of serotypes 3, 4, 6 and 9 (Vreede & Huismans, 1994; Iwata *et al.*, 1992; Williams *et al.*, 1998; Venter, 1999). Solid lines represent the positions of peptides 4–11.

ELISA reactivity of fusion phages

Fig. 5 summarizes the positions of the fusion peptides selected in panning by the four different antibody preparations relative to the amino acid sequence of VP2. Selection by panning may, however, not always reflect binding in other systems. The antigenicity of selected examples of the fusion peptides was therefore also examined in ELISAs in which the fusion phages were immobilized by adsorption to the surface of plastic microtitre wells and allowed to react with the total anti-AHSV chicken IgY. Thirteen clones displaying a series of peptides (1, 2, 4–12 and 14; see Figs 1–4) were chosen to represent each of the VP2 antigenic regions. The clone displaying peptide 7 (Fig. 1*c*) was the best binder in ELISA (Fig. 6*a*). Its fusion peptide overlapped peptide 6 (Fig. 1*c*), with the residues HKAEVKKFL being common to both. Peptides with common overlapping residues did not, however, always react

identically in ELISA. This is illustrated by the aforementioned peptides 6 and 7, where peptide 7 gave an absorbance of 0·923 compared with 0·414 for peptide 6. This contrasts with the overlapping peptides 9 and 10 (Fig. 1*d*), which produced signals very similar to each other. When the sequences of the most-strongly reactive serotype 3 peptides tested with chicken antibodies in ELISA were aligned with the sequences of VP2 of three other AHSV serotypes, all were found to correspond to regions showing inter-serotype variability (Fig. 6*b*). Neither the MAb nor the horse IgG bound with sufficient affinity to any of their cognate fusion peptides to produce convincing ELISA readings. Nevertheless, the MAb 2F2 recognized all the fusion phage that it had selected from the random epitope library, with peptide X4 consistently producing the highest signal (Table 1). In this assay, the different phages used as antigens were adsorbed to the microtitre plate wells as described for Lib-VP2.

In order to show that ELISA signals resulted from a specific interaction between fusion peptide and antibody and to determine reaction conditions that could be used later to deplete neutralizing antibodies, an attempt was made to inhibit the interaction. Phage clones displaying peptides representing a region of VP2 that produced an ELISA signal of at least four times the background were selected for this experiment, in which the chicken antibodies were cross-absorbed with an excess of phage prior to ELISA. Fig. 6(*a*) shows that the A_{492} values could be reduced effectively to background levels by homologous clones. Under these same conditions it was, however, not possible to reduce the neutralizing activity measurably of anti-AHSV immune IgY (not shown).

Discussion

By using three different approaches to maximize diversity, a composite gene-targetted filamentous phage library (Lib-VP2) was constructed that displayed peptides of between five (Fig. 4*c*) and 55 (Fig. 4*a*) residues representing fragments of the gene encoding VP2 of AHSV-3. Comparing the peptides selected from Lib-VP2 by four different antibody preparations with the amino sequence of the entire protein revealed several antigenic sites, most of which were in the N-terminal half of the protein. In some regions, the selected peptides were grouped in clusters with overlapping tracts, e.g. IRRA, HKAEVKKFL and QGTRTAAIVET. Others were represented by individual peptides, e.g. ARWVEW (Fig. 1). With a MAb, the minimal overlap between more than one peptide can indicate the essential residues accommodated by its paratope (Fack *et al*., 1997). With a polyclonal population, however, it is highly probable that some antibodies also recognize adjacent regions. For instance, despite HKAEVKKFL being common to peptides 6 and 7 (Fig. 1*c*), the signal produced by peptide 7 in ELISA with the chicken IgY was more than twice that of peptide 6 (Fig. 6). Nevertheless, native VP2 is unlikely to have extensive linear sequences exposed on its surface. If this were so, it is improbable that the panned peptides would have clustered in well-defined regions. This finding is in accordance with the notion that, although the entire accessible surface is potentially antigenic, very few continuous sequences are found on the surface of most globular proteins (Barlow *et al*., 1986; Van Regenmortel, 1990). The C-terminal portions of VP2 of AHSV serotypes 3 (L. Bentley, unpublished), 4 (Martínez-Torrecuadrada & Casal, 1995) and 9 (Venter, 1999) have been found to be non-antigenic in immunoblotting. Accordingly, phage display failed to identify highly antigenic regions within the 494 C-terminal residues of serotype 3. While this does not preclude residues in this region from being part of a discontinuous epitope, a lack of antigenic reactivity may also result if this part of the polypeptide is involved in interactions with neighbouring capsid proteins.

When Lib-VP2 was panned with either chicken IgY affinity-purified on denatured protein or with antibodies eluted from whole virus, the selected peptide clusters corresponded closely, identifying three major antigenic regions between residues 324 and 543 (Figs 1 and 2). Antigenicity in these regions was not eliminated by reduction, SDS denaturation and binding to a membrane. Operationally, these sequences therefore represent continuous epitopes. Unlike the electroblot-purified antibodies, however, the virus-specific preparation eluted from intact virus did not select any peptides corresponding to amino acids 228–282 (Fig. 1*a*, peptide 2). This sequence, although only selected once by the electroblot-purified antibodies, bound the anti-AHSV IgY in ELISA (Fig. 6). Hence, it may represent a cryptotope. The original AHSV used as immunogen could conceivably have contained some free or possibly even proteolytically degraded VP2 that gave rise to antibodies that bound epitopes that are normally hidden. The horse immunoglobulins also selected a single phage clone displaying this sequence (peptide 3, Fig. 4*a*). This was not surprising, since cryptotopes could be revealed to the horse immune system during uncoating or before assembly.

Apart from peptides 2 (Fig. 1*a*) and 3 (Fig. 4*a*) and the one near the C terminus, the chicken and horse antibodies selected sequences that differed in their minimal overlapping residues. Nevertheless, with both the horse and the chicken antibodies, it is clear that significant antigenicity resides in the region encompassed by amino acids 393–432. The horse antibodies bound a set of peptides that were located within the overall antigenic region stretching from residues 224 to 543, but some sequences identified as being highly antigenic with the chicken antibodies (e.g. the regions depicted in Figs 1*b* and *d* and 2*a* and *c*) were not identified at all. It is possible that, owing to intrinsic genetic differences, the equine immune system recognized a different set of epitopes. Horse antibodies have, for example, been reported not to be able to recognize dodecameric synthetic peptides reflecting VP5 sequences (Martínez-Torrecuadrada *et al*., 1999) in PEPSCAN and to show epitope-boundary frame shifts and differences in immunodominance when compared with mouse and human

antisera (Atassi *et al*., 1996). It may also be significant that the chicken was immunized with non-replicating AHSV together with an adjuvant. The horse serum, on the other hand, was from a host animal infected with cell culture-derived inoculum. The immune responses to AHSV could therefore have involved differences in antigen presentation, resulting in different cytokine profiles, which in turn could affect the clonal expansion of B or T helper cells.

While most of the fusion peptides selected from Lib-VP2 were clearly antigenic, a sequence ARWVEWA (Fig. 1*e*) near to the N terminus was recognized by IgY that had been eluted from a truncated VP2 from which these residues were absent. Logically, therefore, it cannot be a true epitope. A very similar sequence nearer the middle of the polypeptide chain, namely WVDW (residues 428–432), was selected with the horse immunoglobulins (Fig. 4*b*). WVEWA is therefore a potential internal antigenic mimic (Fehrsen & Du Plessis, 1999). Surprisingly, the MAb 2F2 also panned a peptide that included these residues. It is therefore not clear whether they represent an epitope, a mimotope or an adventitious binding sequence. WVEWA was, however, not highly antigenic in ELISA.

Two other clones were selected by the neutralizing MAb 2F2. The only correspondence with any of the other peptides was with peptide 12 (Fig. 3*b*), which overlapped the end of peptide 11 (Fig. 2*c*) by ten residues. None produced any significant readings in ELISA. When the same MAb was used to pan the XCX_{15} random-epitope library, peptides that included the sequences LEKW (Table 1, peptide X7) and MYPFTY (Table 1, peptides X1, X2 and X3) were selected. Recognizably similar sequences occur near the C terminus (MYPVHY) and at the N terminus (LEKWRDL) of the MAb-selected peptide 1 (Fig. 3*a*). These residues may therefore form part of a discontinuous epitope. The XCX_{15} peptide (sequence X7) that included the amino acids LEKW produced the stronger ELISA signal (Table 1), indicating a greater contribution towards binding. On the phage, the peptide that includes LEKW is located between two cysteine residues and therefore has the potential to form a loop structure, which may assist it in mimicking the authentic epitope. No similarities with the other peptides selected by the MAb (Fig. 3, peptide 12) were found, and it probably represents background binding.

ELISA was used to test the binding of representative phage-displayed peptides under conditions different from those under which they were selected. In general, the strongest ELISA signals correlated with antigenic regions characterized by clusters of panned peptides. With the chicken IgY, ELISA signals could be reduced by up to 90% with homologous phage preparations. Nonetheless, under similar conditions, the neutralizing activity of the chicken antibodies could not be reduced (not shown). With AHSV-4, bacterially expressed fragments of VP2 identified a domain extending from residues 253 to 413 that evoked neutralizing antibodies in mice but, like the phage-displayed fusion peptides, could not reduce serum neutralizing capacity (Martínez-Torrecuadrada & Casal, 1995). Neutralizing activity was localized further to residues 283–379 and 379–413 of the serotype 4 polypeptide. These regions fall within the overall antigenic region encompassing amino acids 324–543 of AHSV-3 (Fig. 5). None of the peptides identified by phage display, however, was shown to be involved directly in neutralization.

A comparison of the aligned amino acid sequences of four AHSV serotypes revealed that a tract of residues encompassing peptide 7, the sequence that bound strongest in ELISA, varied significantly between four different AHSV serotypes. This peptide, selected with chicken IgY, included a sequence also recognized by the horse antibodies. In the development of serotype-discriminatory immunoassays, the antigenicity of this region, combined with sequence variability, makes it a potentially worthwhile subject for further investigation. Another potentially useful region is included in peptide 11. The horse serum used for panning was, however, not a good binder in ELISA with any of the fusion phages that were tested. The bacteriophage fUSE2 displays at most five or six copies of the fusion peptide per phage (Specthrie *et al*., 1992). The use of synthetic peptides or other fusion proteins could, therefore, conceivably enable a higher epitope density to be achieved in ELISA, possibly resulting in more efficient assays. It may nevertheless be necessary to pan epitope libraries with more than one horse immune serum in order to locate regions recognized consistently by equine antibodies.

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