Antigenic Profile of African Horse Sickness Virus Serotype 4 VP5 and Identification of a Neutralizing Epitope Shared with Bluetongue Virus and Epizootic Hemorrhagic Disease Virus

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African horse sickness virus (AHSV) causes a fatal disease in horses. The virus capsid is composed of a double protein layer, the outermost of which is formed by two proteins: VP2 and VP5. VP2 is known to determine the serotype of the virus and to contain the neutralizing epitopes. The biological function of VP5, the other component of the capsid, is unknown. In this report, AHSV VP5, expressed in insect cells alone or together with VP2, was able to induce AHSV-specific neutralizing antibodies. Moreover, two VP5-specific monoclonal antibodies (MAbs) that were able to neutralize the virus in a plaque reduction assay were generated. To dissect the antigenic structure of AHSV VP5, the protein was cloned in *Escherichia coli* using the pET3 system. The immunoreactivity of both MAbs, and horse and rabbit polyclonal antisera, with 17 overlapping fragments from VP5 was analyzed. The most immunodominant region was found in the N-terminal 330 residues of VP5, defining two antigenic regions, I (residues 151–200) and II (residues 83–120). The epitopes were further defined by PEPSCAN analysis with 12mer peptides, which determined eight antigenic sites in the N-terminal half of the molecule. Neutralizing epitopes were defined at positions 85–92 (PDPLSPGE) for MAb 10AE12 and at 179–185 (EEDLRTR) for MAb 10AC6. Epitope 10AE12 is highly conserved between the different orbiviruses. MAb 10AE12 was able to recognize bluetongue virus VP5 and epizootic hemorrhagic disease virus VP5 by several techniques. These data will be especially useful for vaccine development and diagnostic purposes.   © 1999 Academic Press

INTRODUCTION

African horse sickness virus (AHSV) is an arthropod-borne orbivirus of the family *Reoviridae* and is the causal agent of African horse sickness (HAS), characterized by a high mortality in horses and other equids. To date, nine serologically distinct AHSV serotypes (AHSV-1 to AHSV-9) have been identified with no evident cross-neutralization among them. The disease is confined to sub-Saharan Africa, although periodic epizootics have caused severe outbreaks of the disease outside enzootic regions, i.e., North Africa, the Middle East, and southern Europe (Rodriguez *et al.,* 1992; Mellor, 1993). AHSV virions possess a double-stranded RNA genome of 10 discrete segments, each encoding one to two proteins (Roy *et al.,* 1994), encapsidated by two distinct protein shells. The outer layer of the virus is composed of two proteins, VP2 and VP5, whereas the inner capsid is formed by two major polypeptide chains, VP3 and VP7, enclosing three other minor proteins, VP1, VP4, and VP6.

AHSV-4 VP5 is a 56-kDa protein, rich in nonpolar amino acids (Iwata *et al.,* 1992). The bluetongue virus

(BTV) VP5 has a globular structure (Hewat *et al.,* 1992). Yang and Li (1993) reported N-linked glycosylation of BTV VP5; however, it has been shown that AHSV-9 VP5 is not N-linked glycosylated (du Plessis and Nel, 1997). Although present in the outer shell of the virus particles, VP5 is mostly unexposed on the surface of the virus. Despite this, it is one of the earliest serologic markers in AHSV infections (Martinez-Torrecuadrada *et al.,* 1997). Its biological function remains unknown. Orbivirus VP5 proteins are relatively conserved, with 45% identity between AHSV-4 and BTV-10 and 43% identity between AHSV-4 and epizootic hemorrhagic disease virus-1 (EHDV-1), with identity rising to 64% when amino acids of similar character are considered (Roy *et al.,* 1994). If VP5 sequences from different AHSV serotypes are compared, 90% of the amino acids are similar, of which 81% are identical, between AHSV-4 VP5 and AHSV-9 VP5 (du Plessis and Nel, 1997), and 91% of similar amino acids and 83% of identical amino acids are shared between AHSV-4 and -6 VP5 (Williams *et al.,* 1998).

It has been shown that AHSV capsid protein VP2 elicits serotype-specific antibodies (Bremer *et al.,* 1990) and is a major target for the neutralizing response of the host (Ranz *et al.,* 1992; Burrage *et al.,* 1993; Martínez-Torrecuadrada *et al.,* 1994; Martínez-Torrecuadrada and Casal, 1995). The presence of neutralizing epitopes ex-
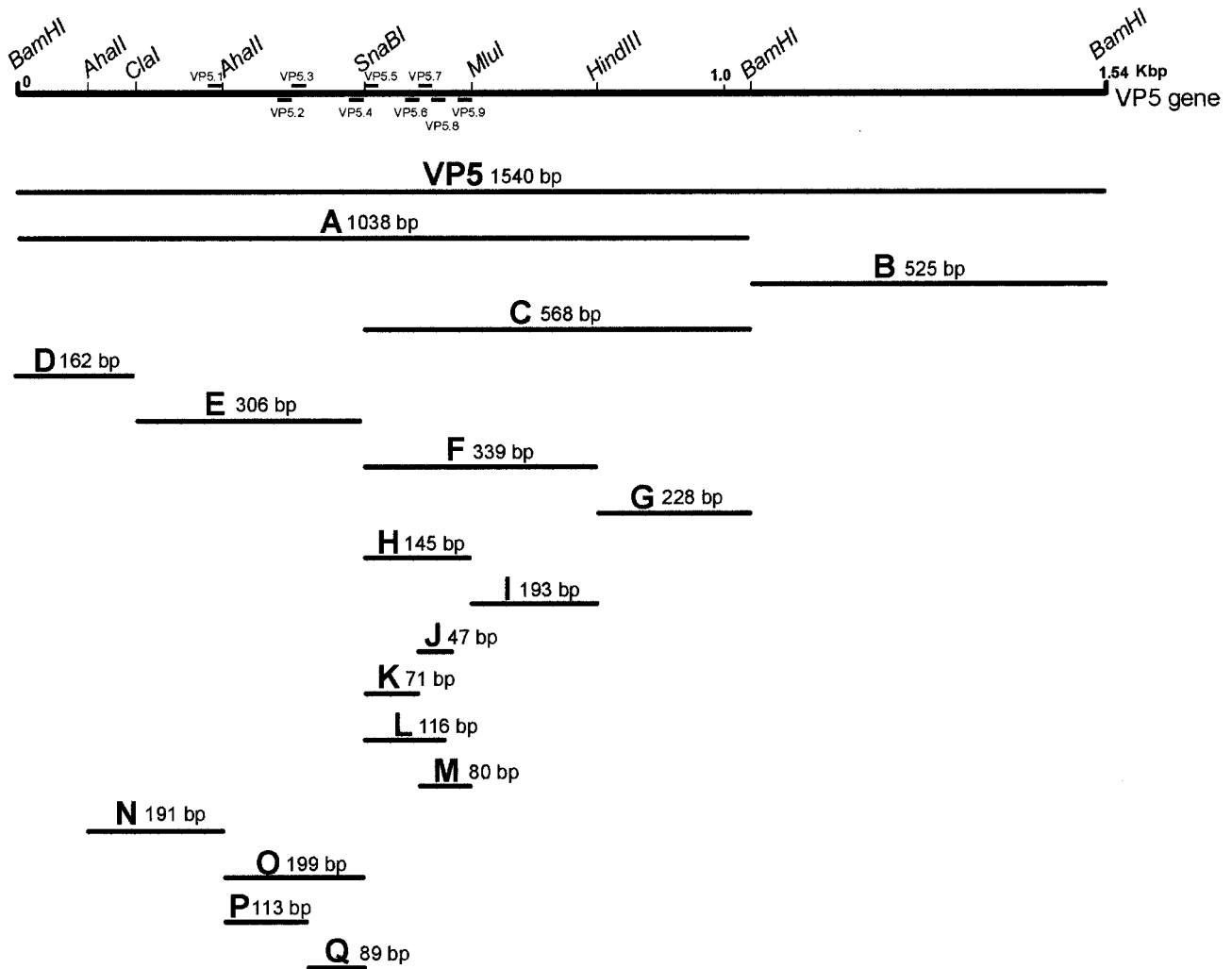
FIG. 1. Schematic representation of AHSV-4 VP5 fragments. The fragments were constructed as described under Materials and Methods using appropriate restriction enzymes sites or PCR. The VP5 gene is represented by a darker line showing the restriction enzyme sites in italics and the primers for PCR are shown as small bars with the corresponding names used in the fragmentation process. Fragment names and sizes (bp) are indicated above each fragment.

clusively on VP2 has also been well established in other members of orbivirus genus, such as BTV (Huismans and Erasmus, 1981; Grubman *et al.,* 1983; Marshall and Roy, 1990) and Palyam serogroup viruses (Whistler and Swanepoel, 1990), except for Kemerovo serogroup viruses like Broadhaven virus (BRDV), in which the epitopes responsible for neutralization were located on the corresponding VP5 protein (Moss *et al.,* 1987). However, it has not been described previously that both proteins of the outer capsid of an orbivirus contain epitopes responsible for virus neutralization. The role of VP5 in neutralization has not yet been clearly assigned. Previous results have shown that the presence of VP5 together with VP2 in BTV and AHSV can enhance the immune response, despite the fact that antibodies raised against purified or recombinant BTV VP5 alone do not neutralize BTV infectivity (Marshall and Roy, 1990; Roy *et al.,* 1990). Recently, we have shown that the best confor-

mation of VP2 to expose the immunodominant neutralization epitopes was achieved when coexpressed with VP5 in the baculovirus system. Moreover, the use of these proteins together with VP7 induced a complete protective immune response in horses (Martínez-Torrecuadrada *et al.,* 1996). Current AHS vaccines contain modified live virus or inactivated virus. Although these vaccines are quite protective, their use can present some disadvantages, such as possible reversion to virulence by mutation or genome segment reassortment with circulating virus, incomplete attenuation or inactivation, the requirement of handling large amounts of live virus, and the difficulty in distinguishing vaccinated and infected animals. Therefore, the development of a noninfectious, safe subunit virus vaccine would be of fundamental importance.

The mapping of antigenic determinants, especially neutralizing epitopes, is essential in order to learn more

about virus neutralization and to identify the critical epitopes required for the development of a subunit or synthetic vaccine and diagnostic reagents. In this report, we have used a collection of recombinant proteins and synthetic peptides to delineate eight antigenic sites on VP5 of AHSV-4. These were determined by analyzing neutralizing antibody samples from different sources (monoclonal antibodies (MAbs) and horse and rabbit polyclonal antisera) for their affinity to recombinant fragments and synthetic peptides of AHSV-4 VP5 (see Fig. 1).

RESULTS

African horse sickness virus neutralization induced by VP5

To investigate the immunogenicity of the baculovirus-expressed AHSV-4 VP5 and to determine whether or not AHSV-4 VP5 is able to elicit antibodies that neutralize homologous virus, rabbits were immunized with baculovirus-infected cell extracts containing recombinant VP5. Antibodies to recombinant VP5 reacted with the virion by ELISA, reaching titers higher than 10^4. Likewise, by Western blot analysis the anti-VP5 serum was shown to give
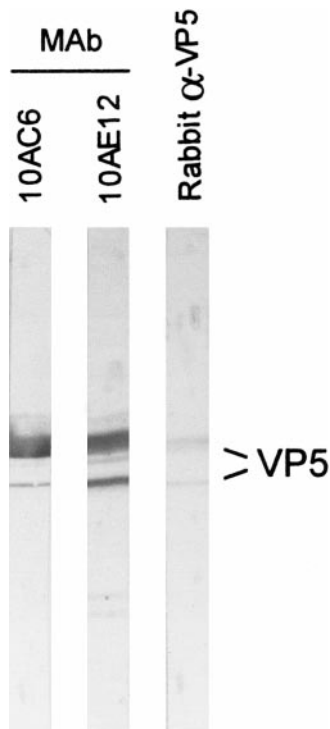

FIG. 2. Recognition in Western blot analyses of virion VP5 by AHSV-4 VP5-specific MAbs and rabbit anti-VP5 serum. Proteins from purified AHSV-4 were separated on SDS–9% polyacrylamide gel, transferred onto a nitrocellulose membrane, and cut into strips. The strips were incubated with the corresponding MAb or rabbit serum as indicated on the top of each strip, and reaction was detected using phosphatase-conjugated anti-mouse or protein A for rabbit immunoglobulins using NBT and BCIP as substrates. The migrations of VP5 and truncated forms are indicated as VP5 on the right-hand side.

TABLE 1

Neutralization Activity Elicited by VP5

Serum	Neutralization titer[a]
Rabbit α-VP5	256
Rabbit α-VP2	1,280
Horse α-AHSV-4	10,240
Rabbit preα-VP5	<5

[a] Neutralization titers represent the reciprocal of the highest serum dilution that causes 50% reduction in plaque-forming units, in a Vero monolayer protection assay. Sera from rabbits immunized with recombinant VP2 and an experimentally infected horse were included as positive controls. Preimmune rabbit serum was used as negative control. Sera were inactivated for 30 min at 56°C to remove complement.

positive signals with viral VP5 and truncated forms of VP5, albeit at low levels as shown in Fig. 2.

The ability of the rabbit anti-VP5 serum to neutralize AHSV-4 *in vitro* was determined by a monolayer protection assay in the absence of complement (Table 1). VP5 was able to induce neutralizing antibodies at titers of 10^2. Rabbit anti-VP2 serum and AHSV-4 infected horse serum used as positive controls showed titers of 10^3 and 10^4, respectively. As expected, the preimmune rabbit serum did not contain any AHSV-4 neutralizing activity. These data confirm that VP5 contains neutralizing epitopes for AHSV and can elicit a significant neutralizing response albeit at titers that were significantly lower than those induced by VP2.

Production of AHSV VP5-specific monoclonal antibodies

After seven fusions only two MAbs (10AC6 and 10AE12) that were positive to VP5 by ELISA and immunoblotting (Fig. 2) were produced. However, both failed to immunoprecipitate VP5 (data not shown). The characteristics of the two MAbs are summarized in Table 2. MAb 10AC6 was IgG2a, whereas 10AE12 was IgG1. A competitive binding ELISA was developed in order to determine whether 10AC6 and 10AE12 react with distinct antigenic sites. 10AC6 and 10AE12 did not compete for antigen binding, suggesting that they recognize different epitopes and therefore define two antigenic sites on VP5, further discussed as sites I and II. These results suggested that epitopes recognized by both MAbs would be linear and might become exposed after denaturing conditions.

The functional relevance of the epitopes recognized by both MAbs 10AC6 and 10AE12 was tested *in vitro* by a plaque reduction assay. Surprisingly both MAbs exhibited neutralizing activity; MAb 10AE12 was able to reduce the plaque-forming units (PFU) number of AHSV-4 up to 80%, and MAb 10AC6 was able to reduce it to a somewhat lesser extent (Table 2). Ascitic fluid of a highly neutralizing AHSV-4 VP2-specific MAb, 8CH7, was used

TABLE 2

Summary of AHSV-4 VP5 MAb Characterization

Hybridoma	Isotype	Antigenic site	IP	Western blot	ELISA titer[a]	Neutralization[b]
10AC6	IgG2a	I	—	+++	0.1 μg/ml	68%
10AE12	IgG1	II	—	+++	0.1 ng/ml	80%

[a] ELISA titers were expressed as the concentration of purified IgG giving three times the blank value.
[b] Neutralizing activity was measured as the percentage of inhibition of viral infectivity in a plaque-forming units reduction test.

as a positive control and reduced the viral infectivity by more than 98%. A negative control did not neutralize.

Expression of recombinant VP5 fragments in *Escherichia coli*

The AHSV-4 VP5 gene and 17 overlapping fragments of the VP5 protein were cloned in pET3- or pET3x-derived vectors, which were designated pET3b-VP5, pET3b-ΔA, pET3c-ΔB, pET3b-ΔC, pET3xb-ΔD, pET3xa-ΔE, pET3xb-ΔF, pET3xa-ΔG, pET3xb-ΔH, pET3xb-ΔI, pET3xa-ΔJ, pET3xa-ΔK, pET3xa-ΔL, pET3xa-ΔM, pET3xb-ΔN, pET3xb-ΔO, pET3xc-ΔP, and pET3xa-ΔQ. After a 3-h induction, the corresponding recombinant proteins generated were analyzed by Coomassie blue staining of SDS–PAGE (Fig. 3). It was remarkable that all the fragments containing the amino terminus of the molecule (pET3b-VP5, pET3b-ΔA, and pET3xb-ΔD) were extremely toxic to *E. coli,* causing a significant decrease in the growth rate of the cells and leading to rapid cell lysis after induction with 0.4 mM IPTG. These plasmids were viable only in the presence of pLysE, resulting in a very low level of expressed fragment, since cells carrying this plasmid accumulate substantial levels of lysozyme that reduce the basal level of T7 RNA polymerase.

Fragments that did not contain the VP5 amino terminus were expressed at high levels in the bacterial cells as shown in Fig. 3. In most cases, a band was observed with a size in agreement with the expected molecular mass according to the coding capacities of the expressed fragment. Fragments J and P migrate abnormally. This artifact could be explained by a high proportion of charged residues in these fragments (Takano *et al.,* 1988). Although the fusion proteins were insoluble, large quantities could be readily enriched and partially purified by solubilization with 4 M guanidinium hydrochloride.

Epitope mapping with recombinant VP5 fragments

The location of antigenic epitopes was determined in the different *E. coli*-derived VP5 fragments by immunoblotting analyses with MAbs and antisera from infected horses and immunized rabbits (Table 3). As expected, the whole VP5 was recognized by all the antibodies used. VP5 was initially divided into fragments A and B.

Fragment A was expressed at very low levels, making it difficult to assess its reactivity with antibodies. Fragment B was extensively synthesized; however, it did not react with the MAbs or the antisera, indicating a very low antigenicity. Therefore, epitope mapping was focused on the larger part of the molecule. Fragment A was divided in three portions, C, D, and E. Fragment C reacted with MAb 10AC6, with horse antisera, and slightly with the anti-VP2VP5 rabbit sera. Fragment D, which contains the N-terminus of the protein, was clearly recognized by horse and rabbit anti-VP2VP5 sera, despite its low ex-
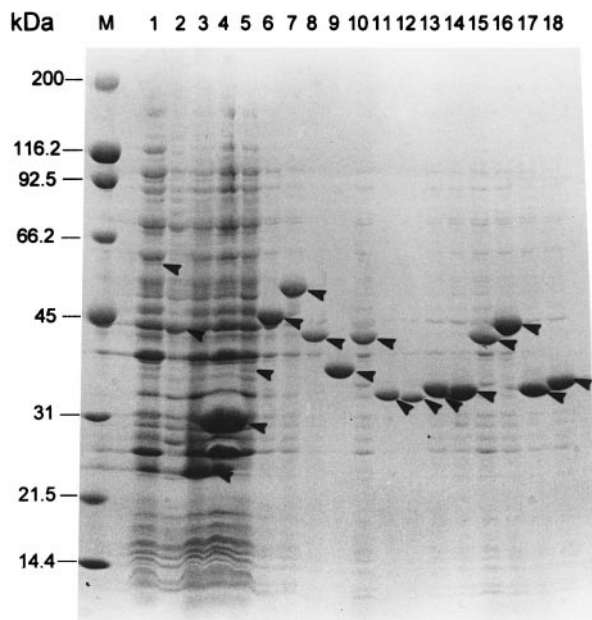

FIG. 3. SDS–PAGE analysis of the expression of the AHSV-4 VP5 fragments in *E. coli* (strains BL21(DE3)pLysS or BL21(DE3)pLysE). Bacteria containing the recombinant pET3- or pET3x-derived plasmids were induced for 3 h with 0.4 mM IPTG. After treatment with loading buffer and boiling for 5 min, proteins were separated on a 7–15% SDS–polyacrylamide gel and stained with Coomassie blue. Each line represents bacterial lysates transformed with the following expression vectors: lane 1, pET3b-VP5; lane 2, pET3b-ΔA; lane 3, pET3c-ΔB, lane 4, pET3c-ΔC; lane 5, pET3xb-ΔD; lane 6, pET3xa-ΔE; lane 7, pET3xb-ΔF; lane 8, pET3xa-ΔG; lane 9, pET3xb-ΔH; lane 10, pET3xb-ΔI; lane 11, pET3xa-ΔJ; lane 12, pET3xa-ΔK; lane 13, pET3xa-ΔL; lane 14, pET3xa-ΔM; lane 15, pET3xb-ΔN; lane 16, pET3xb-ΔO; lane 17, pET3xc-ΔP; lane 18, pET3xa-ΔQ. Molecular weight markers (M) are given in kDa on the left-hand side. Positions of fusion proteins are indicated with arrowheads.

TABLE 3

Reactivity of VP5 Fragments by Immunoblotting with Horse and Rabbit Antisera

Fragment	Amino acids	Mab 10AC6	Mab 10AE12	Horse α-AHSV-4	Rabbit α-VP2VP5
VP5	1–505	+++	+++	++[a]	++
A[b]	1–341	+	+	−	+
B	341–505	−	−	−	−
C	151–341	+++	−	++	+
D[b]	1–49	−	−	+	+
E	48–150	−	+++	+++	+++
F	151–265	+++	−	+++	+++
G	264–341	−	−	+	++
H	151–200	+++	−	+++	+++
I	200–265	−	−	+++	++
J	174–189	−	−	−	++
K	151–174	−	−	−	−
L	151–189	−	−	−	−
M	174–200	−	−	+	−
N	21–84	−	−	+++	+++
O	85–150	−	+++	+++	+++
P	83–120	−	+++	++	++
Q	121–150	−	−	+	+

[a] −, no reaction; +, positive reaction (from +++, maximum to +, slight reaction).

[b] Extremely low expression of these fragments due to toxicity for bacteria.

pression. No MAb gave a positive reaction with this fragment. Finally, fragment E reacted strongly with horse antisera, rabbit anti-VP2VP5 sera, and MAb 10AE12. To further define the epitopes, regions C and E were divided in two segments each: F and G, and N and O, respectively. Polyclonal antisera reacted intensely with fragments F, N, and O and, to a lesser extent, with fragment G. MAb 10AC6 reacted exclusively with fragment F and MAb 10AE12 recognized fragment O.

To further locate the epitope defined by MAb 10AC6, fragment F was split into two halves, H and I, through the *Mlu*I restriction site present at nt 616. Only fragment H (49 aa) was recognized by MAb 10AC6. Four overlapping polypeptides covering the length of fragment H were then expressed in *E. coli,* resulting in fragments J, K, L, and M. Surprisingly, none of these fragments were able to react with 10AC6. These fragments were also tested with the polyclonal sera. Horse sera reacted with fragments H, I, and M and rabbit anti-VP2VP5 sera recognized fragments H, I, and J.

To define the 10AE12 epitope, two polypeptides included in fragment O were also constructed, yielding fragments P and Q. MAb 10AE12 recognized fragment P, but not Q. Fragment P also gave a stronger reaction to polyclonal sera than did fragment Q.

In summary, two major antigenic domains corresponding to fragments E and F were identified on the N terminus of VP5. By successive fragmentation of these regions and expression in *E. coli,* the antigenic site I,

recognized by MAb 10AC6, was located between aa 151 and 200, and the antigenic site II, defined by MAb 10AE12, was situated between aa 83 and 120.

Immunogenicity of VP5-derived fragments

In an attempt to investigate whether or not *E. coli*-derived fragments, comprising the neutralizing domains I and II, were able to elicit neutralizing antibodies, mice were immunized with purified fragments E and H, containing 10AE12 and 10AC6 epitopes, respectively. After the mice were immunized three times, serum samples were tested for their ability to neutralize the virus *in vitro* by a monolayer protection assay. However, none of the sera showed any detectable neutralization activity, despite the fact that they were able to react with the AHSV-4 virion by ELISA and with the corresponding peptides by PEPSCAN analyses (data not shown).

Epitope mapping with synthetic peptides

PEPSCAN analysis of the VP5 sequence was used to define linear antigenic domains and thus to delineate more precisely the binding specificity of MAbs 10AE12 and 10AC6, rabbit antisera to recombinant AHSV4 VP5 and VP2/VP5, or horse antisera to AHSV-4. Eight antigenic sites were identified with the rabbit antisera and the two MAbs. They were numbered 1 through 8, according to their order of occurrence from the N-terminus (Fig. 4). The amino acid sequences corresponding to the peptides involved in these sites are displayed in Table 4. Considering the height of the absorbances at the antigenic sites, the MAbs exhibited higher avidity than the rabbit sera, and rabbit antiserum to VP2/VP5 was of higher titer than rabbit anti-VP5 serum. Some antibody samples share the same antigenic sites: site 4 for MAb 10AE12 and rabbit anti-VP2/VP5; site 6 for rabbit sera to VP5 and to VP2/VP5. Sites 1, 3, 4, 6, 7, and 8 detected by PEPSCAN with rabbit antiserum to VP2/VP5 agreed quite well with the reactivity of the recombinant fragments in Western blot analysis (Tables 3 and 4), especially the regions between residues 27 and 98 (sites 1, 3, and 4) and between 211 and 232 (site 7), which are most antigenic. The equine sera did not exhibit any binding to the peptides.

The surface accessibility of these sites on the virus was studied by blocking antibody binding with purified AHSV-4. Whereas no surface exposure of the mapped linear antigenic sites could be shown for the two MAbs, the binding by rabbit polyclonal antibodies to antigenic sites 2, 3, 4, 6, 7, and 8 could be blocked by more than 50% with respect to a control absorption with an irrelevant virus. Surprisingly, binding to antigenic site 4 could be blocked using rabbit antiVP2/VP5 serum, but hardly at all using the MAb 10AE12. This suggests that the MAb has a greater affinity to the linear sequences displayed by the synthetic peptides than by the virus, suggesting
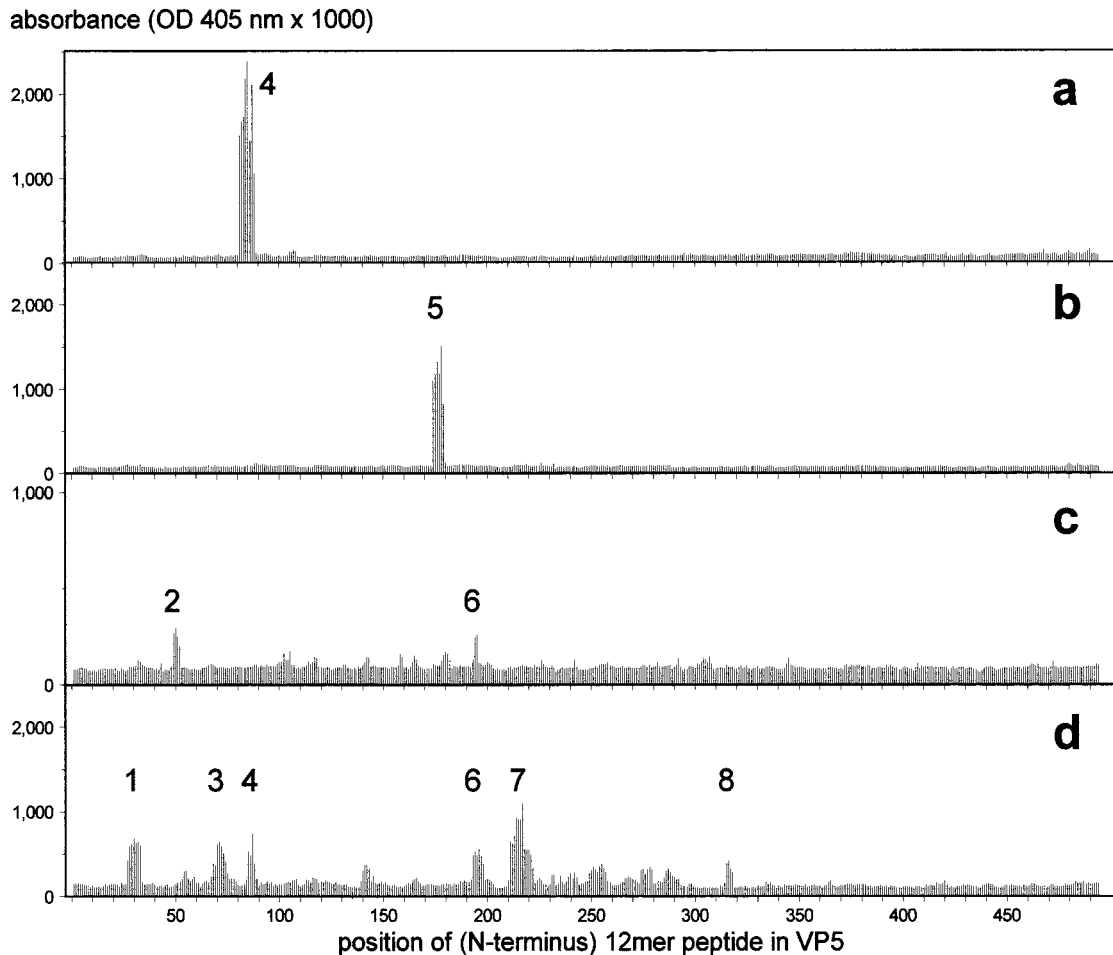
absorbance (OD 405 nm x 1000)

FIG. 4. Mapping of antigenic sites with 12-mer peptides using PEPSCAN analysis. Antigenic sites detected in four antibody samples were numbered 1–8, from N- to C-terminus. a–d represent the results obtained using MAb 10AE12, MAb 10AC6, rabbit anti-VP5, and rabbit anti-VP5/VP2 sera. The sequences involved in these antigenic sites are displayed in Table 4.

that the recognition site of MAb 10AE12 at the epitope level is not fully exposed on the surface of the virus. In contrast, the polyclonal rabbit antiVP2/VP5 serum has a greater affinity for the intact virus. These results indicate that the epitopes recognized by the MAb and the polyclonal antisera, although identical at the amino acid sequence level must be different at the atomic level, which defines the epitope–paratope interaction (Van Regenmortel, 1998). Nevertheless, the blocking of antibody binding by native protein or virus as shown in these experiments is indicative of surface exposure.

Conservation and cross-reactivity of epitopes between orbiviruses

The conserved nature of the antigenic sites that bind to MAbs 10AE12 and 10AC6 was studied between the viral family members AHSV, BTV, EHDV, and BRDV. Alignment of cognate VP5 sequences showed that MAb 10AE12 recognizes a highly conserved epitope in orbiviruses (Fig. 5). The sequence of site II, or antigenic site 4 according to the PEPSCAN data, PDPLSPGE is present

in all the VP5 sequences from different serotypes of BTV and EHDV. There is only a single substitution in AHSV-9 VP5, $Ser^{88} \rightarrow Asn$. However, this is a conservative change since both amino acids are polar. BRDV VP5, which is more distantly related, showed two substitutions, $Ser^{88} \rightarrow Asn$ and $Gly^{90} \rightarrow Val$. On the other hand, for the 10AC6 epitope, only two of seven amino acids are conserved among the orbiviruses analyzed. These results were confirmed by Western blot analysis, since MAb 10AE12 was able to recognize baculovirus-derived VP5 from BTV-10 and EHDV-1 (Fig. 6), while MAb 10AC6 reacted only with AHSV-4 VP5 (data not shown).

DISCUSSION

VP5 is a protein present in the outer shell of the orbivirus particle (Roy, 1992), whose biological role remains to be clarified. To date, it has been assumed that antibodies against orbivirus VP5 do not neutralize infectivity (Mertens et al., 1989; Roy et al., 1990; Marshall and Roy, 1990). This does not seem to be true for AHSV, as we have demonstrated here that recombinant VP5 ex-

TABLE 4

Antigenic Sites Identified by PEPSCAN on AHSV-4 VP5

Site[a]	Antibody	Sequence[b]	Position in VP5	% Blocking by AHSV-4[c]
1	Rabbit αVP2/VP5	MYKLAGKTLQRVVESEVG	27–44	38
2	Rabbit αVP5	DGVMQGAIQSIIQGE	49–63	100
3	Rabbit αVP2/VP5	KQAVILNVAGTLESAP	70–85	64
4	10AE12	LESAPDPLSPGEQLLYNKV	81–99	8
4	Rabbit αVP2/VP5	PDPLSPGEQLLYNK	85–98	93
5	10AC6	RALQTEEDLRTRDETRM	174–190	0
6	Rabbit αVP2/VP5	EYREKFDALKEAIEIE	193–208	65
6	Rabbit αVP5	YREKEDALKEAIE	194–206	27
7	Rabbit αVP2/VP5	ATHDEAIQEMLDLSAEVIETAS	211–232	86
8	Rabbit αVP2/VP5	TQHVIDAVLPIVK	315–327	96

[a] Site numbers correspond to those assigned in Fig. 4.
[b] The complete peptide sequence of an antigenic site is given. The underlined amino acids represent the common sequence present in all the peptides of that antigenic site.
[c] Blocking of antibody binding by purified AHSV-4 was calculated as a percentage as described under the PEPSCAN section of Materials and Methods. The control samples with the ADV material yielded comparable peak heights as in the untreated samples analyzed in Fig. 4.

pressed in insect cells and used for rabbit immunization was able to elicit a significant neutralizing activity. Moreover, two mouse MAbs obtained after immunization with virions were also able to neutralize AHSV and their binding specificity was mapped to the small peptide level. This is the first report of such neutralizing ability associated with an orbivirus VP5. Since no three-dimensional structure is currently known for AHSV, correlations of antigenic differences between AHSV and BTV and the structure of their outer capsid proteins cannot be determined. It is likely that structural differences between the two viruses may account for some of the differences in immunological behavior.

The antigenicity of the capsid protein VP5 of AHSV serotype 4 appears to be mostly located in its N-terminal 330 residues and more specifically inside fragments E and F, spanning residues 48–265. In fact, when the antigenicity was mapped at the peptide level, most of the eight linear antigenic sites defined by PEPSCAN (sites 2 to 7) were mapped in this area of the VP5. Antigenic sites in AHSV VP5 were defined by using mouse MAbs and rabbit antisera. The use of horse sera did not allow the

FIG. 5. Alignment of the amino acid sequences of VP5 from different orbiviruses between residues 85 and 92 and residues 179 and 185 corresponding to epitopes recognized by MAbs 10AE12 and 10AC6, respectively. The sequences of the orbivirus VP5s are available from the GenBank database under the following accession numbers: AHSV-4 VP5, M94731; AHSV-6 VP5, AF021237; AHSV-9 VP5, U74489; BTV-1S VP5, M36713; BTV-2A VP5, X62283; BTV-10 VP5, D12532; BTV-11 VP5, U03284; BTV-13 VP5, X54308; BTV-17 VP5, X55359; EHDV-1 VP5, X55782, and BRDV VP5, M58030. The conserved amino acids are indicated by asterisks.
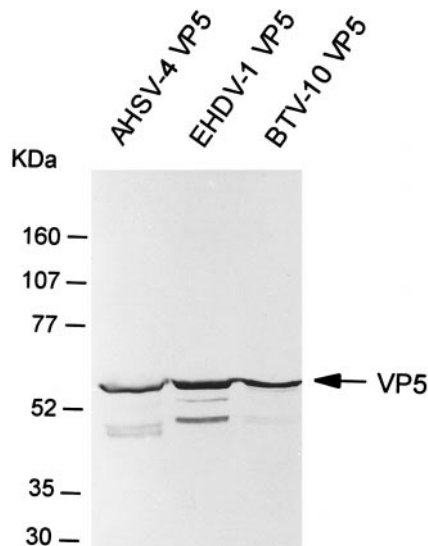

FIG. 6. Reactivity of MAb 10AE12 with orbivirus VP5s by Western blot. Crude extracts of Sf9 cells infected with recombinant baculoviruses AcAHSV-4.5, AcEHDV-1.5, or AcBTV-10.5 expressing AHSV-4 VP5, EHDV-1 VP5, and BTV-10 VP5, respectively, were prepared, mixed with protein sample buffer, and loaded onto a 9% SDS–polyacrylamide gel. Fractionated proteins were transferred to a nitrocellulose filter and incubated with MAb 10AE12 as described under Materials and Methods. The arrow indicates the migration of the recombinant orbivirus VP5s. Molecular weight markers are indicated on the left-hand side.

characterization of any antigenic site in VP5 at the amino acid level, although immunoblotting reactivity with the fragments did define the same immunodominant regions E and F and several subareas inside these two. The absence of detecting binding of horse sera to VP5 peptides is a puzzling observation. The most likely explanation is that the length of the epitopes recognized by the horse sera is longer than the 12-mers used in the PEPSCAN analysis. In fact, recent data in our laboratory support this hypothesis, as horse sera were able to recognize a 18-mer peptide based on the 10AE12 epitope (VP5 residues 81–99) in an indirect ELISA (Martínez-Torrecuadrada, unpublished results). This is one of the limitations of the PEPSCAN technology, which has been shown previously for other epitopes (Kusters et al., 1989). These results suggest that horse antibodies mainly recognize longer linear epitopes or discontinuous epitopes resulting from a combination of sequences from different proteins (VP2, VP5, and/or VP7).

Antigenic site 4, defined by MAb 10AE12, has several interesting properties: (i) it is able to induce neutralizing antibodies; (ii) it is well conserved, not only among the AHSV serotypes, but also among the different orbiviruses (BTV, EHDV, BRDV); and (iii) antibodies to this site are able to recognize VP5 proteins from BTV and EHDV by immunoblotting, which suggests that MAb 10AE12 can be used as a group-specific reagent, suitable for the detection of any orbivirus infection. This is the first report of such a reagent for use with orbiviruses. Further development of this MAb, and the corresponding synthetic peptide, for diagnostic applications is under investigation.

However, antisera from mice immunized with fragment E, which contains the 10AE12 epitope, were unable to neutralize the virus. The conserved sequence probably reflects the lack of exposure of the epitope on the virus surface and, probably, an important structural role for this sequence. The lack of exposure was confirmed by the failure of this MAb to immunoprecipitate VP5 and by the absorption experiments with full virions in PEPSCAN. Since the MAbs were generated by immunization with intact AHSV-4 virions, this site must have become exposed during denaturation, disassembly, and/or processing of the virus in the target animal. However, MAb 10AE12 is able to bind to VP5 on purified AHSV-4 in an ELISA assay (Table 2), suggesting that the corresponding epitope is not deeply buried, because the small conformational changes induced in the particles when coated on ELISA plates are enough to improve its accessibility.

BTV VP5 epitope mapping (Wade-Evans et al., 1988; Yang et al., 1992; Wang et al., 1995) described several antigenic determinants also located in the N-terminal half of the molecule. One of these was a linear epitope located between residues 175 and 189 (Yang et al., 1992), which corresponds very closely with site 5 defined by

MAb 10AC6 in AHSV. Another group has identified the region bound by the BTV-specific MAb 30F12, using phage display, between residues 142 and 183 (Wang et al., 1995). The antigenic region (aa 175–189) described by Yang et al. (1992) seems to be exposed on the surface of the virion in five US BTV serotypes, which appears contradictory to our results regarding the accessibility of the epitope for 10AC6. This could be due to minor variations in the surfaces of both orbiviruses. In any case, it is clear that this region is a hot immunogenic spot in the VP5 of orbiviruses. The amino acid sequence at this region is well conserved among BTV serotypes, but very poorly conserved between orbiviruses (Fig. 5). It also corresponds to a very hydrophilic region of the VP5 in both BTV and AHSV. Surprisingly, MAb 10AC6, which recognizes this epitope, was found to be weakly neutralizing. In contrast, antisera from mice immunized with E. coli-derived fragments containing the epitope did not show detectable neutralizing titer, although they reacted strongly with the peptide as demonstrated by PEPSCAN analysis (data not shown).

No antigenic regions were found in the C-terminal part of VP5, as no antibodies of any origin, including horse, react with this region. Sequence comparisons with BTV, EHDV, and BRDV showed that this region is relatively well conserved among the different orbiviruses, suggesting that it is probably not exposed and may be involved in the preservation of the virus structure. A similar situation was described for AHSV-4 VP2 (Martínez-Torrecuadrada and Casal, 1995). We can thus speculate that the C-terminal regions of both proteins are interacting to support the outer shell structure, but are inaccessible to the immune system.

In conclusion, an immunodominant region, between residues 48 and 265, and eight different antigenic sites have been mapped in the AHSV-4 VP5. The N terminus of VP5 seems to be the most immunodominant region and may be the most surface-exposed and accessible to antibodies area in VP5. In contrast to the VP5 proteins from other orbiviruses, AHSV VP5 alone is able to induce neutralizing antibodies albeit at lower levels than VP2. Since the defined epitopes are bound by neutralizing MAbs and one of them is conserved among different orbiviruses, the amino-terminal region of VP5 could be of special interest for inclusion in a new generation of vaccines for AHSV and diagnostic tests for orbiviruses.

MATERIALS AND METHODS

Cells and viruses

AHSV-4 was grown and assayed in confluent monolayers of a permanent line of Vero cells (ATCC CCL 81) in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 5% fetal calf serum (FCS) and antibiotics. AHSV virions were purified as described previously (Martínez-Torrecuadrada et al., 1994). The Spodoptera

frugiperda cell clone Sf9 (ATCC CRL1711) was used to propagate recombinant baculovirus AcAHSV-4.5 and to express AHSV-4 VP5 (Martínez-Torrecuadrada *et al.,* 1994). Also, AcBTV-10.5 (Marshall and Roy, 1990) and AcEHDV-1.5, kindly provided by P. Roy (IVEM. Oxford. UK), were used to express BTV-10 VP5 and EHDV-1 VP5, respectively.

E. coli strains DH5 and XL1-Blue were used for cloning of target DNA into pET vectors (Studier *et al.,* 1990). *E. coli* strains BL21(DE3)pLysS and BL21(DE3)pLysE (Grodberg and Dunn, 1988) were used as the host for expression. *E. coli* strain GM119, lacking the *dam* methylase, was used to prepare nonmethylated plasmid DNA for subsequent digestion by restriction enzyme *Cla*I.

Production of MAbs

Production and selection of hybridoma lines using purified AHSV as antigen were performed following the procedure previously described (Sanz *et al.,* 1985). The culture medium of hybridomas was screened for reactivity with AHSV by ELISA. The protein specificities of ELISA-positive clones were determined by immunoprecipitation and immunoblotting (Ranz *et al.,* 1992). VP5-positive hybridomas were cloned four times by limiting dilution and purified from ascitic fluid by HPLC (Beckman). The isotype of the MAbs was determined by ELISA, using specific anti-mouse subtype antisera (Sigma).

Construction of *E. coli* expression vectors

The complete AHSV-4 VP5 coding sequence was obtained from the recombinant plasmid pAcYM1.AHSV-4.6 (Martínez-Torrecuadrada *et al.,* 1994). The constructs used in this study are shown in Fig. 1. The following VP5 fragments were generated by using convenient restriction enzymes sites: VP5 gene, A (nt 1–1038, *Bam*HI fragment, nt 1 is the first nucleotide (G) of the segment M6); B (nt 1039–1564, *Bam*HI fragment); C (nt 470–1038, *Sna*BI to *Bam*HI); D (nt 1–162, *Bam*HI to *Cla*I); E (nt 163–469, *Cla*I to *Sna*BI); F (nt 470–809, *Sna*BI to *Hind*III); G (nt 810–1038, *Hind*III to *Bam*HI); H (nt 470–615, *Sna*BI to *Mlu*I); I (nt 616–809, *Mlu*I to *Hind*III); N (nt 78–269, *Aha*II fragment); O (nt 270–469, *Aha*II to *Sna*BI). Fragments with *Bam*HI ends were directly ligated with *Bam*HI-digested phosphatase-treated pET3 or pET3x (Studier *et al.,* 1990). pET3x vectors were chosen to express fragments smaller than 350 bp. Fragments with *Bam*HI-incompatible ends were first subcloned into pMTL plasmids to generate *Bam*HI-compatible ends and subsequently cloned into pET expression vectors.

If appropriate enzymes sites were not available, VP5 fragments were synthesized by PCR with Vent DNA polymerase (Biolabs). The samples were subjected to 25 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 1.5 min. The oligonucleotides used were VP5.1, 5′-CAT<u>AGATCT</u>CGGCGCCAGACCCGTTGAGC-3′; VP5.2, 5′-AAA<u>GGATCC</u>TTCTATTTTCGCATTGTGTG-3′; VP5.3, 5′-GCG<u>AGATCT</u>GAAAAATTTGGTAAAGATTTAT-3′; VP5.4, 5′-CTA<u>GGATCC</u>GTACTTAATTTCGTTACCTTCC-3′; VP5.5, 5′-ATG<u>GGATCC</u>GTAGCAAAAGCGCTTACC-3′; VP5.6, 5′-TTG<u>AGATCT</u>GCGATATAGCTTTGTAATG-3′; VP5.7, 5′-CTA<u>AGATCT</u>CGCGCGTTACAAACAGAGGA-AGATTTG-3′; VP5.8, 5′-CTA<u>GGATCC</u>TCTAGTCTCATCTC-GTGTGCGCAAATCTTC-3′; and VP5.9, 5′-TTC<u>GGATCC</u>-CGCGTCAAATTTCTCTCTAT-3′ (restriction sites are underlined). The VP5 fragments generated by PCR were the following: J (nt 539–586 from primer VP5.7 to VP5.8), K (nt 470–541 from primer VP5.5 to VP5.6), L (nt 470–586 from primer VP5.5 to VP5.8), M (nt 539–619 from primer VP5.7 to VP5.9), P (nt 266–379 from primer VP5.1 to VP5.2), and Q (nt 380–469 from VP5.3 to VP5.4). The resulting PCR products were digested with *Bam*HI or *Bgl*II and were cloned directly into *Bam*HI-digested and dephosphorylated pET3x.

After transformation of *E. coli* DH5 competent cells, positive clones were selected by extensive restriction analysis of plasmid. The orientation of the inserts and the junction sequences of the recombinant plasmids were sequenced by the dideoxynucleotide method, using primers 5′-CTTTAAGAAGGAGATATAC-3′ for pET3- and 5′-CTATCCGCAACGTTATGGGC-3′ for pET3x-derived plasmids. Strain BL21(DE3)pLysS or BL21(DE3)pLysE competent cells were transformed with each of the recombinant plasmids and positive colonies were selected by their resistance to ampicillin and chloramphenicol.

Induction, purification, and analysis of *E. coli*-derived fusion proteins

Single colonies of BL21(DE3)pLysS or BL21(DE3)pLysE containing the indicated plasmid were grown and induced with 0.4 mM IPTG (Boehringer Mannheim) as described (Martinez-Torrecuadrada and Casal, 1995). After 3 h, the induced bacteria were centrifuged for 5 min at 10,000 rpm and dissolved in loading buffer (10 mM Tris–HCl, pH 6.9, 10% SDS, 10% β-mercaptoethanol, 0.02% bromophenol blue, 25% glycerol). The mixture was boiled for 5 min and electrophoresed in a 7–15% SDS–polyacrylamide gradient gel. Visualization of proteins was carried out by staining with Coomassie brilliant blue.

Inclusion bodies containing the VP5-fragment fusion proteins were purified by guanidinium chloride solubilization as described previously (Martínez-Torrecuadrada and Casal, 1995).

Immunoblotting analyses

After SDS–PAGE, proteins were transferred electrophoretically onto a Hybond-C membrane (Amersham, UK) and blocked with 3% skimmed milk in PBS containing 0.05% Tween 20 for 1 h at room temperature. After blocking, filters were incubated with either rabbit sera at

a 1:100 dilution, horse sera at a 1:50 dilution, or purified MAb IgG at 10 μg/ml in blocking buffer for 2 h at room temperature and washed several times with 0.05% Tween 20 in PBS. Bound antibody was detected with a 1/2000 dilution of alkaline phosphatase-conjugated protein A (Sigma), peroxidase-conjugated protein G (Pierce, Rockford, IL), or phosphatase-conjugated anti-mouse IgG (Sigma), respectively. Finally, membranes were developed with 4-chloro-naphthol and hydrogen peroxide in PBS for the detection of peroxidase activity or with nitroblue tetrazolium chloride (Gibco BRL) and bromo-chloroindoyl phosphate (Pierce) as substrates for detection of phosphatase activity.

Immunization of animals

Groups of five 3-week-old BALB/c mice were primed intraperitoneally with about 50 μg of the corresponding fusion protein, which was semipurified as described above and emulsified with an equal volume of Freund's complete adjuvant (Sigma). Two further injections, by the same route and with the same dose of antigen in Freund's incomplete adjuvant (Sigma), were given after 2 and 6 weeks. Each group of mice was bled and antisera were collected 10 days after the final injection.

Female New Zealand White rabbits were immunized with baculovirus-expressed VP5. AcAHSV-4.5-infected insect cells were harvested at 72 h postinfection by centrifugation at 200 g for 10 min, washed once with PBS, and resuspended in PBS at a density of $5-7 \times 10^7$ cells/ml. An aliquot was analyzed by SDS–PAGE and Coomassie blue staining to estimate the amounts of recombinant VP5 present. A total of 2×10^7 infected cells, containing about 50 μg of VP5, were injected intramuscularly using Alhydrogel (Superfos, Denmark) together with 100 μg of Quil A (Superfos) as adjuvants. Rabbits were boosted twice at 4-week intervals, first with adjuvant and then without adjuvant. Blood samples were taken for serological assays 10 days after the final immunization. Another group of female New Zealand White rabbits were immunized with purified recombinant VP2 and VP5 as described previously (Martínez-Torrecuadrada et al., 1994).

Polyclonal anti-AHSV-4 sera were obtained from infected horses or from horses vaccinated with inactivated vaccine (Martínez-Torrecuadrada et al., 1994).

ELISAs

An indirect ELISA was used to detect AHSV-specific antibodies. Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 0.5 μg/well of CsCl gradient-purified AHSV-4 in 0.1 M carbonate buffer, pH 9.6. Plates were washed and serial fivefold dilutions of rabbit or mouse sera in blocking buffer (0.35 M NaCl, 0.05% Tween 20 in PBS) were then added. After a 1-h incubation at 37°C, plates were washed and to

each well was added a 1/2000 dilution of peroxidase-conjugated anti-mouse (Sigma) or anti-rabbit IgG (Sigma) in blocking buffer and incubated 1 h at 37°C. Plates were washed again and bound enzyme was detected by adding ABTS (2,2′-azinobis(3-ethylbenzothiazoline-sulfonic acid)) (Sigma) as substrate. The reaction was stopped after 10 min with the addition of 2% SDS and the absorbance was measured at 405 nm using an ELISA reader.

Identification of MAb-defined antigenic sites on AHSV-4 VP5 was carried out by using a competitive binding ELISA as described previously (Ranz et al., 1992) with the purified virus as antigen source.

In vitro neutralization assays

To assess the AHSV-4 neutralization capability of antibodies, a PFU reduction test, 50 μl of seed virus, containing about 10^5 PFU, were mixed with an equal volume of each ascitic fluid and incubated for 2 h at 37°C. Serial tenfold dilutions were made with the virus–antibody mixtures and were added to 4×10^4 Vero cells. Virus adsorption was allowed to proceed at 37°C for 2 h. The medium was discarded and cells were covered with agar overlay medium (DMEM containing 1% agarose and 2% FCS). After 7 days at 37°C, cell monolayers were fixed with 20% formaldehyde in PBS and stained with 1.5% crystal violet in 50% ethanol. Plaques were counted in each well. Virus neutralization activity was expressed as the percentage of plaque reduction. Ascitic fluid of neutralizing AHSV-4 VP2-specific and rabbit hemorrhagic disease virus-specific MAb were included as positive and negative controls, respectively.

Vero monolayer protection assay was performed as described previously (Martínez-Torrecuadrada and Casal, 1995). End-point titration was determined as the reciprocal value of the highest serum dilution that causes 50% reduction of cell monolayer.

PEPSCAN

The complete set of 494 overlapping 12-mer peptides with sequences based on that of AHSV-4 VP5 (Iwata et al., 1992) was synthesized onto polyethylene according to established procedures and tested for binding by antibody in an ELISA test as described (Geysen et al., 1984). The criterium for assigning a site as antigenic was taken as follows: the absorbance value should be at least twice the background and there should be two or more neighboring peptides that reach this value. The background was taken as twice the average absorbance value of 20 consecutive low-reacting peptides for which the CV (coefficient of variation = SD/average $\times$ 100) is below 20% of the average value.

The accessibility of the antigenic sequences in AHSV was studied by preabsorbing antisera with sucrose gradient purified AHSV-4 or as a negative control Aujezsky disease virus similarly as previously described (Lange-

veld *et al.,* 1993). The percentage of blocking is calculated from the absorbance values obtained for a site (after substracting the background value) according to the formula $(1 - (EA_{405nm}C - EA_{405nm}AHSV)/EA_{405nm}C) \times 100\%$, where $EA_{405nm}C$ is the sum of absorbance values in control sample and $EA_{405nm}AHSV$ is the sum of absorbance values in AHSV-4 preabsorbed sample.

ACKNOWLEDGMENTS

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