Characterization of neutralization sites on the circulating variant of swine vesicular disease virus (SVDV): a new site is shared by SVDV and the related coxsackie B5 virus

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Using a panel of new monoclonal antibodies (mAbs), five neutralizing, conformation-dependent sites have been identified on the antigenic variant of swine vesicular disease virus (SVDV) circulating currently. In studies on the antigenic conservation of these sites, the four antigenic/genetic groups of SVDV described showed distinguishable patterns, confirming this classification. By sequencing mAb-resistant mutants, the five sites have been mapped precisely and localized on a three-dimensional model of the SVDV capsid. All were found to be orientated, to a different extent, towards the external surface of the capsid. Three of the five sites, located in VP1, VP2 and VP3, correspond to epitopes identified previously in historic isolates as sites 1, 2a and 3b, respectively. Another site, site IV, which maps to position 258 of VP1, corresponds to an epitope reported recently and is described in this study to be specific for isolates of the most recent antigenic group of SVDV. A fifth site is described for the first time and corresponds to the unique neutralizing site that is common to both SVDV and coxsackie B5 virus; it maps to positions 95 and 98 of VP1, but may also include positions nearby that belong to site 1 on the BC-loop of VP1, suggesting the classification of site Ia. These results may have useful diagnostic and epidemiological applications, since mAbs to the new conserved site Ia provide universal reagents for SVDV detection systems, while the specificity of mAbs to site IV make them unique markers for the most recent strains of SVDV.

Introduction

Swine vesicular disease (SVD) is a highly contagious pig disease characterized by symptoms that are indistinguishable from those of foot-and-mouth disease (FMD). SVD was first identified in Italy in 1966 (Nardelli et al., 1968) and many outbreaks have been reported subsequently in Europe and Eastern Asia (Brocchi et al., 1997). The European Union is, nowadays, an area free from SVD, with the exception of Italy, where outbreaks have occurred every year.

SVD virus (SVDV) is a member of the genus Enterovirus, family Picornaviridae, and is a porcine variant of the human pathogen coxsackie B5 virus (CV-B5) (King et al., 2000). The antigenic and molecular relationship between these two viruses has been studied widely (Graves, 1973; Brown et al., 1973; Zhang et al., 1993) and recent data suggest that SVDV is a genetic sub-lineage of CV-B5 that evolved between 1945 and 1965, after the transfer of CV-B5 from humans to pigs (Zhang et al., 1999). No different serotypes have been described, but analysis of the reactivity of SVDV isolates with monoclonal antibodies (mAbs) has allowed the distinction of four different antigenic groups, corresponding to four distinct genetic lineages (Brocchi et al., 1997).

To date, seven independent epitopes involved in neutralization have been identified on the SVDV capsid using mAbs that had been raised against European and Asiatic isolates from the 1970s. These isolates belong to the second chronologically occurring antigenic group. Six of these epitopes could be grouped into three main sites (Kanno et al., 1995; Nijhar et al., 1999), which are analogous to the antigenic sites defined previously in poliovirus. Site 1, close to the fivefold axis in the three-dimensional (3D) model, is composed of two epitopes, one including residues 87–88 in the BC-loop of VP1 and the second including residue 261 at the C terminus of VP1 (Lin et al., 1998). Site 2 comprises two separate epitopes...
Table 1. Anti-SVDV mAbs used and characterized in this study

The 14 mAbs produced against SVDV Italy 92 (this study) and five further mAbs that cross-reacted with SVDV Italy 92 (Brocchi et al., 1997), obtained previously against other strains, are shown. Reactivity with SVDV Italy 92 was determined by ELISA and VN and WB assays. No viral protein reacted with the mAbs in WB assays.

<table>
<thead>
<tr>
<th>mAb</th>
<th>mAb isotype</th>
<th>Eliciting SVDV strain</th>
<th>Reactivity with SVDV Italy 92</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A10</td>
<td>IgM</td>
<td>Italy 72</td>
<td>2.8</td>
</tr>
<tr>
<td>4H3*</td>
<td>IgM</td>
<td>Italy 72</td>
<td>2.8</td>
</tr>
<tr>
<td>5B7*</td>
<td>IgG2b</td>
<td>Italy 72</td>
<td>2.8</td>
</tr>
<tr>
<td>1A2</td>
<td>IgM</td>
<td>Italy 91</td>
<td>3.8</td>
</tr>
<tr>
<td>2H8</td>
<td>IgG1</td>
<td>Italy 91</td>
<td>0.9</td>
</tr>
<tr>
<td>3D8</td>
<td>IgG1</td>
<td>Italy 92</td>
<td>4.3</td>
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<tr>
<td>3D10</td>
<td>IgG2a</td>
<td>Italy 92</td>
<td>4.3</td>
</tr>
<tr>
<td>2A12</td>
<td>IgG2a</td>
<td>Italy 92</td>
<td>4.8</td>
</tr>
<tr>
<td>2H12</td>
<td>IgG2a</td>
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<td>4.8</td>
</tr>
<tr>
<td>1F8</td>
<td>IgG1</td>
<td>Italy 92</td>
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<td>2E10</td>
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<tr>
<td>1D3</td>
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<tr>
<td>5C4</td>
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<td>2G6</td>
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<tr>
<td>1B3</td>
<td>IgG2a</td>
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</tr>
<tr>
<td>5H6</td>
<td>ND</td>
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<td>0.6</td>
</tr>
<tr>
<td>3G9</td>
<td>IgG2b</td>
<td>Italy 92</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* mAbs mapped previously by Nijhar et al. (1999): 4H3, site 3B (amino acid 73 of VP3); 5B7, site 2A (amino acid 163 of VP2).
† End-point titres of supernatant of hybridoma cultures (log_{10} reciprocal dilution giving optical density values > 0.2).
‡ Average neutralizing titres expressed as reciprocal log_{10} are given in parentheses.
§ These mAbs were used as ascites fluids; titres ranged between 15- and 400-fold higher than those of the corresponding supernatants of hybridoma cultures.
ND, Not determined.

of neutralization, 2a and 2b, both of which are located on the EF-loop of VP2 and involve residues 163 and 154, respectively. Site 3 has been described as a complex site and involves amino acids on three structural proteins; it is composed of epitopes 3a (comprising residues 272 and 275 of VP1 and residue 60 of VP3) and 3b, which combines residues 233 of VP2 (HI-loop) and residues 73 and 76 of VP3 (BC-loop) of one pentamer with residue 70 of VP2 (BC-loop) from an adjacent pentamer. The seventh epitope, located in the C terminus of VP3 (residue 234), is a neutralization site typical of SVDV and has never been recognized in other picornaviruses (Nijhar et al., 1999). All these sites were predicted to be conformational and exposed to the surface.

Very recently, using mAbs specific for the most ancient isolate of SVDV, Italy 66, two further neutralizing epitopes located in VP1 have been reported, one formed by residues 258 and 266 and the second formed by residue 225 (Rebel et al., 2000).

In this work, we describe the characterization of a new panel of neutralizing mAbs raised against an isolate of SVDV (isolated in Italy in 1992) that represents the most recent antigenic variant, first detected in Europe in 1992 (Brocchi et al., 1997) and which is still circulating (Borrego et al., 2000a). The reasons for the production of a panel of new mAbs were to study the antigenic evolution of SVDV that has occurred during recent years and for the potential improvement of SVD diagnosis. To correctly apply mAbs to these fields of study, their preliminary characterization is essential. Results of this characterization have allowed us to define five neutralizing sites on the SVDV capsid, one of which is described for the first time.

Methods

**Viruses.** SVDV isolates used in this work are the Italian isolates that represent the four antigenic groups (Brocchi et al., 1997): Italy 66 (first group), Italy 72 (isolate R178, second group), Italy 91 (isolate R1046,
Table 2. Primers used for PCR amplification and/or sequencing of the capsid-coding region (polyprotein P1) of the MAR mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5′ → 3′)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>615D</td>
<td>615–638</td>
<td>CATATTGTATTGGATTGCCCACC</td>
<td>PCR-L, sequencing</td>
</tr>
<tr>
<td>1715R</td>
<td>1693–1715</td>
<td>GCAGTCCATTATATTCCGCCAC</td>
<td>sequencing</td>
</tr>
<tr>
<td>2051D</td>
<td>2051–2070</td>
<td>TATGCCCATGGTCCGCCAG</td>
<td>PCR-R, sequencing</td>
</tr>
<tr>
<td>2143R</td>
<td>2119–2142</td>
<td>GGTGACTATGCTAGCAAGAC</td>
<td>PCR-L, sequencing</td>
</tr>
<tr>
<td>2802R</td>
<td>2780–2802</td>
<td>GTAACTCCAGATCAAATCTTGC</td>
<td>sequencing</td>
</tr>
<tr>
<td>3317R</td>
<td>3317–3340</td>
<td>TGAATGGCCGACGTACAGCAACC</td>
<td>PCR-R</td>
</tr>
<tr>
<td>3502R</td>
<td>3503–3525</td>
<td>TCAATGTGACTGGATGCTT</td>
<td>PCR-R</td>
</tr>
</tbody>
</table>

third group) and Italy 92 (isolate R1072, fourth group). In addition, the enterovirus CV-B5 (isolate 1954/UK/85), received from the Pirbright Laboratory, UK, was included in some assays. Viruses were propagated in IBRS-2 cell monolayers and harvested when cytopathic effect (CPE) was maximal. The viruses used for mice immunization were inactivated with acetylethylenimine (Brown et al., 1963) and purified by centrifugation through sucrose gradients, as described previously (Seechurn et al., 1990).

- **mAbs.** BALB/c mice were immunized with 2–4 doses of 30 µg each of purified SVDV Italy 92 at intervals of 1 month. Cell fusion and cloning of positive hybridomas were performed following standard procedures (Brocchi et al., 1993; Galfre & Milstein, 1981). Selection was based on results of a trapping ELISA against the homologous virus. Also, other mAbs obtained previously against different SVDV variants, but which cross-reacted with the most recent variant, were included in this study (Table 1) (Brocchi et al., 1997).

- **ELISA.** The assay used for both screening hybridomas and antigenic characterization of SVDV was a trapping ELISA (Brocchi et al., 1997). Briefly, ELISA microplates were coated with a saturating concentration of anti-SVDV rabbit serum and pre-titrated concentrations of viruses (supernatant of infected cells) were trapped. Each mAb was then incubated with the virus prior to the addition of anti-mouse peroxidase-labelled immunoglobulins (lg). End-point titres of mAbs were determined as the reciprocal dilution that gave optical density values higher than 0.2, while the reactivity of mutants with each mAb was expressed as a percentage of the corresponding reaction with the parental strain, assumed to be 100%.

- **Western blot (WB) analysis.** Hybridoma supernatants were assayed against purified SVDV resolved by 10% SDS–PAGE and transferred onto nitrocellulose filters following standard procedures (Harlow & Lane, 1988).

- **Virus neutralization (VN) assay.** Neutralizing activity of mAbs was determined in microplates against 100 TCID₅₀ of SVDV in IBRS-2 cells, as described previously (Brocchi et al., 1995). Neutralizing titres were expressed as the reciprocal log₅₀ of the final dilution of antibody required to neutralize 50% of the inoculated culture.

- **Selection of mAb neutralization-resistant (MAR) mutants.** Samples (50 µl) of serial tenfold dilutions of the parental SVDV Italy 92 (isolate R1072, 10⁶.5 TCID₅₀/ml) were mixed with 50 µl of ascites fluid at two fixed dilutions (1:20 and 1:100) in wells of microplates. Then, a 100 µl volume of IBRS-2 cells at 10⁵ cells/ml in growth medium was added to each well. After incubation for 2–3 days at 37 °C, 5–10 wells for each mAb showing CPE at the highest dilutions of virus were subcultured twice in 24-wells plates in the presence of ascites fluid diluted 1:20.

A fourth passage without mAb was performed and presumptive mutants were harvested when CPE was evident (24–48 h post-infection). After ensuring the lack of reactivity against the selecting mAb by trapping ELISA, four MAR mutants for each mAb were amplified and analysed further.

- **Sequencing.** RNA extracted from the supernatant of infected cultures, as described by Chomczynski & Sacchi (1987), was used as the template for RT–PCR. Reverse transcription was carried out using AMV reverse transcriptase with a hexanucleotide mixture of all possible sequences (‘Random’ primer) (Boehringer Mannheim). The cDNA produced was used as the template for PCR amplification of the region encoding the structural proteins using the primers described in Table 2. Polyprotein P1 was amplified in two fragments, P1-L and P1-R, running from positions 615–2142 and 2051–3525 [numbering according to Inoue et al. (1989)], respectively, which included also some flanking nucleotides from both the 5′ non-translated region (NTR) and protein 2A. Sequencing of PCR fragments and sequence analysis were carried out using an ABI 310 Automatic Sequencer and LaserGene software.

- **Location on a 3D model of the SVDV capsid.** The construction of a 3D model for SVDV Italy 92 (isolate R1072) capsid proteins and location of the identified neutralization sites was carried out as described previously (Jiménez-Clavero et al., 2000). The accessibility of some single residues was determined using the DSSP program (Kabsch & Sander, 1983).

### Results

**Characterization of mAbs against SVDV Italy 92**

A total of 14 mAbs was produced against SVDV Italy 92, isolate R1072, which represents the most recent antigenic
**Fig. 1.** Pattern of reactivity in ELISA of SVDV MAR mutants and reference viruses with the panel of neutralizing mAbs. MAR mutants are named according to the mAb used to select them. For each mAb, four independent MAR mutants were selected; reactivity is expressed as a percentage of binding with respect of the parental strain of SVDV, Italy 92, assumed to be 100%, and is shown in cells as follows: white, reactivity below 10%; light grey, reactivity between 10 and 50%; dark grey, reactivity between 50 and 70%; black, reactivity over 70%. Each cell is subdivided in four sub-cells corresponding to the reactivity of single mutants. In the lower part panel, the reactivity of reference SVDV isolates representing the four known antigenic groups and CV-B5 is shown. In this case, each cell corresponds to the reactivity of single isolates expressed as a percentage of the homologous reaction for each mAb.

**Selection of MAR mutants and identification of neutralization sites**

For each of the 11 strongly neutralizing mAbs, MAR mutants that were able to grow in the presence of a concentration of antibody 10^3–10^4-fold higher than that necessary to neutralize 100 TCID_{50} of the parental virus were obtained.

With the exception of mAbs 3D10 and 3D8, the frequency of mutants resistant to the different mAbs in the parental population ranged from 10^{-3.7} (mAb 1B3) to 10^{-6.3} (mAb 2A12), as estimated by the residual infectious titres observed in the presence of each mAb compared with the titre of the parental virus (data not shown). In contrast, mutants resistant to mAbs 3D10 and 3D8 could only be selected after passages in the presence of sub-neutralizing concentrations of mAbs (ascites fluid dilution 1:20000 to 1:50000), which probably allowed the enrichment of mutants in the initial population.

For each mAb, four MAR mutants, selected independently and grown, were analysed for their reactivity with the 11 mAbs by trapping ELISA. With few exceptions, the reactivity patterns of the four independent mutants were identical and allowed the definition of five main antigenic sites, named sites Ia, Ib, II, III and IV (Fig. 1).

Other neutralizing mAbs (1A2, 2H8, 5A10, 4H3 and 5B7) (Table 1) raised against two previous SVDV variants were used for determining the antigenic profile of the escape mutants and could be assigned to three of the five sites.

Except for sites Ia and Ib, which were associated, but not in a reciprocal way, the other sites were independent of each other, as a mutation in one site did not alter the reactivity of the others.

Site Ia was defined by mAbs 3D8, 3D10 and 2A12: mutations induced in each of the target epitopes affected the reactivity of the three mAbs. However, some MAR mutants (mAb 2A12) were found to maintain high levels of reactivity with the other two mAbs.

Site Ib was defined by mAbs 2H12 and 1F8, together with mAbs 1A2 and 2H8. Site Ib seemed to be associated partially...
A new conserved neutralizing site on SVDV

Fig. 2. Neutralization resistance pattern of SVDV MAR mutants with mAbs raised against SVDV Italy 92. mAbs are grouped into antigenic sites, as defined by ELISA. The neutralizing titres of mAbs against 100 TCID₅₀ of each mutant virus (one representative mutant for each mAb) were calculated and differences in mAb neutralizing titres to the parental (SVDV Italy 92, isolate R1072) and each mutant virus are expressed in cells as follows: white, difference > 2 log₁₀ units; grey, difference between 1 and 2 log₁₀ units; black, difference < 1 log₁₀ unit.

to site Ia, as mutations in site Ia also altered the binding of site Ib mAbs, but not vice versa.

Site II was defined by two mAbs, 1G10 and 5B7, whereas site III was defined by six mAbs, including 5A10 and 4H3 from a previous panel. Within this site, mAb 2G6 identified a distinct epitope, since its reactivity remained unaltered in some MAR mutants induced by other mAbs of this site. Finally, site IV was defined by the unique mAb 1B3.

In order to evaluate the correlation between ELISA reactivity and neutralization pattern, one representative mutant for each mAb was tested for its susceptibility to neutralization by each of the 11 mAbs; neutralization profiles are shown in Fig. 2. The neutralizing titres of the mAbs against mutants were compared to those obtained against the parental virus: when the difference was higher than 2 log₁₀ units, mutants were considered to be resistant to neutralization, while for differences lower than 1 log₁₀ unit, mutants were considered to be equivalent to the parental virus. Intermediate differences were interpreted as weak neutralization ability.

Results of these cross-neutralization assays basically overlapped the results obtained by ELISA, providing further evidence of the non-reciprocal association between sites Ia and Ib and of the existence of different epitopes within complex antigenic sites, in particular epitopes 2G6, within site III, and 2A12, within site Ia.

However, some discordance between binding and neutralizing activity was observed: for instance, mAb 2H12 (site Ib) lost the ability to neutralize site III mutants, although binding ability was retained, as shown by ELISA. In the same way, mAb 1B3 (site IV) maintained ELISA reactivity with the MAR mutant 2E10 (site III), but failed to neutralize it. These results demonstrated a relationship between these sites that was not evident by ELISA; however, this was not investigated further.

Evaluation of the antigenic stability of the neutralizing sites

The level of conservation of the neutralizing sites identified by the mAbs was analysed by testing their reactivity in a trapping ELISA, with reference viruses representing each of the four genetic/antigenic groups of SVDV. The related human enterovirus CV-B5 was included also.

The antigenic profile, shown in Fig. 1, confirmed the classification of SVDV into four antigenic groups, which appeared also to be distinguishable with reference to the five sites identified.

In detail, the results demonstrated that antigenic site Ia is conserved in all of the four groups, including the oldest one, represented by the historic isolate SVDV Italy 66. Site Ib is also a conserved site, although the epitope identified by mAb 1F8 showed some variability. mAbs defining site II reacted with the three SVDV variants that have emerged since 1972, but did not recognize the Italy 66 isolate. mAbs of site III were non-reactive with the representative reference virus for the third antigenic group, formed by isolates that emerged in Italy between 1988 and 1992. Finally, the epitope recognized by mAb 1B3, defining site IV, was specific for the most recent variant and was non-reactive against all of the previous isolates.

With regard to CV-B5, results showed that mAbs to site Ia cross-reacted at different levels with the human enterovirus, confirming the high antigenic stability of this neutralizing site. Consistent with ELISA results, these mAbs also neutralized CV-B5, with comparable titres for both SVDV and CV-B5 (data not shown). Other cross-reacting epitopes were found in site III, corresponding to epitopes 2G6, completely conserved and neutralized, and 5C4, only partially recognized. All other sites were SVDV-specific.
Table 3. Amino acid substitutions found in MAR mutants

For each mutant, amino acid substitutions (three-letter code) and relevant positions were deduced by comparing the sequence of the mutant with that of the parental strain. The first residue shown was found in the parental strain, the second (substituted amino acid) was found in the mutant. Possible mixtures of amino acids due to heterogeneity of nucleotides in the corresponding codon are indicated. Antigenic changes are substitutions thought to be involved in site formation; non-antigenic changes are substitutions thought to be unrelated to antigenicity.

<table>
<thead>
<tr>
<th>Antigenic site</th>
<th>MAR mutant</th>
<th>Antigenic amino acid changes</th>
<th>Non-antigenic amino acid changes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mutant 1</td>
<td>Mutant 2*</td>
</tr>
<tr>
<td>Ia</td>
<td>3D8</td>
<td>84 VP1 (Ser → Pro)</td>
<td>84 VP1 (Ser → Pro)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>85 VP1 (Asp → Val)</td>
<td>95 VP1 (Asn → Lys)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95 VP1 (Asn → Lys)</td>
<td></td>
</tr>
<tr>
<td>Ia</td>
<td>3D10</td>
<td>83 VP1 (Asp → Tyr)</td>
<td>83 VP1 (Asp → Gly)</td>
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<td></td>
<td></td>
<td>98 VP1 (Gln → Arg)</td>
<td>95 VP1 (Asn → Lys)</td>
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<td></td>
<td>2A12</td>
<td>95 VP1 (Asn → Ser)</td>
<td>87 VP1 (Asp → Gly)</td>
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<td></td>
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<tr>
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<td>2H12</td>
<td>84 VP1 (Asp → Asp/Gly)</td>
<td>85 VP1 (Asp → Asn)</td>
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<tr>
<td></td>
<td>1F8</td>
<td>84 VP1 (Ser → Tyr)</td>
<td>84 VP1 (Ser → Phe)</td>
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<tr>
<td>II</td>
<td>1G10</td>
<td>163 VP2 (Glu → Glu/Val)</td>
<td>163 VP2 (Glu → Lys)</td>
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<td>III</td>
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<td>76 VP3 (Pro → Gln)</td>
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<td></td>
<td>5C4</td>
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<td></td>
<td>1B3</td>
<td>258 VP1 (Glu → Lys)</td>
<td>258 VP1 (Glu → Lys)</td>
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<td></td>
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</tr>
</tbody>
</table>

* Only regions where changes were found in mutants 1 were selected for sequencing.

Mapping of neutralizing sites

The capsid-coding region of the parental virus, Italy 92 (isolate R1072), and one MAR mutant for each mAb were sequenced and the deduced amino acid sequences compared to identify changes responsible for site mutation.

All 11 mutant viruses showed more than one nucleotide change that led to an amino acid substitution. To discern the amino acid changes actually involved in the antigenic sites, selected genomic regions of a second resistant virus were also sequenced. The amino acid changes found in the two MAR mutants, reported in Table 3, were classified as either an antigenic change, when considered to be involved in site formation, or a non-antigenic change, when thought to be unrelated to the site of neutralization. The latter were clustered in the region 143–214 of VP1 and corresponded to amino acid residues 199, 208, 209 and 214, with positions 143 and 167 substituted less frequently: changes in these positions were detected in different mutants independently on the selecting mAb and, therefore, were interpreted as not relevant to any of the antigenic sites.

The ‘antigenic’ changes found in the MAR mutants enabled us to map the five antigenic sites on the SVDV structural proteins, with the following observations: (i) mutants selected with different mAbs defining one site showed amino acid changes at the same or contiguous residues; however, different amino acids could substitute the same residue and cause similar effects; and (ii) each MAR mutant had a single amino acid substitution involved directly in site antigenicity, except mutants in site Ia, which could show one to three changes simultaneously.

Thus, site I was located in VP1; within it, epitopes for mAbs 2H12 and 1F8, defining site Ib, mapped to positions 84 and 85 of VP1, respectively, while site Ia seemed to be more complex. Resistance to neutralization by the three mAbs defining site Ia (3D8, 3D10 and 2A12) could be achieved by a combination of different amino acid changes at positions between 83 and 87, together with positions 95 and/or 98; thus, site Ia overlaps, or even includes, site Ib, consistent with ELISA and neutralization profiles of MAR mutants, which already suggested a relationship between these two sites. Only one mutant showed a unique amino acid change in this region (position 95).

Site II was found to map to position 163 of VP2. Site III was found to involve two amino acids of VP3 at positions 73, changed in mutant 2G6, and 76, substituted in mutants 1D3,
Fig. 3. Location of neutralizing sites on a 3D model of the SVDV capsid. (a, c) Pentameric and (b, d) protomeric structures of the SVDV capsid shown in a backbone representation. (a, b) outer view; (c, d) lateral view. (e) Fully filled representation of the external view of the pentamer. (a)–(e) The colour code of the five antigenic sites is as follows: red, site Ia (positions 83, 87, 95 and 98 of VP1); green, site Ib (positions 84 and 85 of VP1); magenta, site II (position 163 of VP2); blue, site III (positions 73 and 76 of VP3); yellow, site IV (position 258 of VP1). Note that positions 84 and 85 of VP1, represented as site Ib in green, are also involved in site Ia (see Table 3). (f) Backbone, external and lateral views of the pentameric structure in which only amino acid positions involved in site Ia are colour-coded as red, positions specific for site Ia (positions 95 and 98 of VP1), and green, positions co-involved in site Ib (positions 83, 84, 85 and 87 of VP1). Positions defining all other sites are shown in a uniform grey.
2E10 and 5C4. Site IV, target of mAb 1B3, mapped at position 258 of VP1.

**Location of the neutralizing epitopes onto the 3D model of the SVDV capsid**

Identified epitopes were projected onto a 3D model of the SVDV capsid. To this end, the 3D structure of Italy 92, isolate R1072, was constructed and amino acid positions responsible for antigenic changes in the MAR mutants were located on this new SVDV model.

As shown in Fig. 3, a significant polarization of all positions toward the external face of the pentamer was predicted, as expected for sites involved in neutralization. Residues involved in site I (a and b) form a cluster near the fivefold axis of symmetry in the 3D model; site III is located close to the threefold axis and site II towards the twofold axis at the peripheral boundary of the pentamer. Site IV occurs as an independent site in a central area of the pentamer.

Given the complexity of site Ia, its conformation has been highlighted in Fig. 3(f). Residues involved appear to be grouped into two clusters: positions 83, 84, 85 and 87, part of which also form site Ib, are clearly exposed on the external surface of the capsid and project at the vertices of the icosahedral structure, while positions 95 and 98, distinctive for site Ia, seem to be partially internalized, which is an uncommon feature for neutralizing sites. Accordingly, calculated accessible surfaces of positions 95 and 98 corresponded to 18 and 20 Å², respectively; these values are significantly lower than those deduced for all other residues involved in the other sites, which ranged from a minimum of 45 Å² for position 163 of VP2 (site II) to a maximum of 134 Å² for position 83 of VP1 (Fig. 3e).

Also, residues in VP1 that were classified as non-antigenic changes were located in the 3D model (data not shown). With the exception of residues 199 and 214, the other positions were not exposed.

**Discussion**

mAbs have proven to be a powerful tool for the characterization of SVDV: they have permitted the classification of virus isolates into four antigenic groups, congruent with four distinct genetic clusters (Brocchi et al., 1997), recommended methods for SVD diagnosis are based on the use of mAbs (Brocchi et al., 1995; Chenard et al., 1998; Fallacara et al., 2000) and have provided an invaluable insight into the antigenic structure of the virus, leading to the identification of nine neutralizing epitopes (Kanno et al., 1995; Nijhar et al., 1999; Rebel et al., 2000). mAbs used in these studies had been raised against historic SVDV isolates from 1966, 1972 and 1976, classified in the two oldest antigenic groups and whose antigenic patterns differ from those of the more recent viruses. In this work, we have used a panel of 11 new neutralizing mAbs, raised against an Italian isolate of SVDV (isolated in 1992) and representing viruses that still circulate today, to identify and characterize five neutralizing sites on the most recent antigenic variant. Four of them correspond to antigenic regions identified previously (Kanno et al., 1995; Nijhar et al., 1999; Rebel et al., 2000), while one new site is described for the first time in this study.

Site Ib corresponds to site 1 and maps to the BC-loop of VP1. Several positions from 83 to 88 are involved, as demonstrated by using either escape mutants or field isolates mutated antigenically in site Ib (Borrego et al., 2000a). Although site Ib was shown to be basically common to the four antigenic groups of SVDV, a recent analysis of more than 180 isolates, all classified in the recent antigenic group, showed that this site is subject to minor, but frequent, changes (Borrego et al., 2000a). Site II corresponds exactly to site 2A of the EF-loop of VP2 and is conserved in all SVDV variants, except the most ancient one. Site III is equivalent to site 3B of the BC-loop of VP3. It showed a variation in isolates of the third antigenic group. Nevertheless, it includes a distinct epitope at position 73 (defined by mAb 2G6), which is conserved in the human enterovirus CV-B5.

Site IV was located at position 258 of VP1 on the basis of MAR mutants (mAb 1B3) and additional data from field isolates indicated also that position 266 of VP1 may be involved (Borrego et al., 2000a). The projection of both positions in the 3D model strongly supports their co-involvement in the site: in fact they were found close to each other on the external face of the pentamer (data not shown). Consistently, these positions have been associated recently to a new antigenic site (Rebel et al., 2000), identified on the historic isolate SVDV Italy 66. Our results confirm the existence of this site on isolates of the most recent antigenic group of SVDV; however, mAb 1B3 did not recognize viruses representing the three preceding variants, suggesting that this site was subject to change between the groups. The group-specificity of mAb 1B3 makes it a marker for the recent antigenic group, which includes all SVDV isolates that have emerged since 1992 (Borrego et al., 2000a). The C terminus of VP1 is a very important neutralization region for many picornviruses, harbouring several sites that probably overlap, but which are functionally independent (Mateu, 1995; Usherwood & Nash, 1995). The identification of a new neutralizing site at the C terminus of SVDV VP1 confirms the immunodominance of this region, where other epitopes have been identified already (Kanno et al., 1995; Lin et al., 1998; Nijhar et al., 1999).

Besides these four sites, a fifth one, which has never been identified before, has been demonstrated by mAbs 3D8, 3D10 and 2A12. We named it site Ia due to its partial association with site Ib: in fact, it involves amino acids 83–87, forming site Ia, in association with positions 95 and/or 98 of VP1. When located in the 3D model of the SVDV capsid structure, the two groups of residues that form site Ia were distributed into two separate clusters: positions shared by site Ib are clearly...
exposed on the surface at the vertex of each pentamer and are easily exposed to variability, consistent with antigenic data (Borrego et al., 2000a). In contrast, positions 95 and 98, peculiar to site Ia, appear to be internalized partially and, according to predictions of the secondary structure of the capsid proteins, do not occur in an exposed loop, as do the majority of neutralizing sites, but occur on a short tract connecting two conserved structures (β-strand C and α-helix A). The internalization of residues 95 and 98 in the 3D structure is consistent with the high antigenic stability of site Ia, which was found to be the most stable site, and is conserved in all SVDV antigenic groups and even in the related human pathogen CV-B5. Also, the mechanism of neutralization of SVDV and CV-B5 based on this site must be similar, since the three mAbs were found to neutralize both viruses with the same efficiency.

The finding of one mutant showing a unique change at amino acid 95 suggests that this position is essential for the conformation of site Ia. However, it is likely that, in most cases, substitutions of residues 95 and/or 98 cause structural rearrangements, with an introduction of changes occurring close to positions 83–87, thus co-involving site Ib. Differently, site Ib may change independently, leaving positions 95–98 unaltered.

With the exclusion of sites Ia and Ib, which were demonstrated to interfere with each other, the other neutralizing sites described are substantially independent, proving that antibodies may use multiple sites and probably multiple mechanisms of neutralization. However, our neutralization results suggested possible relationships between different sites, with reflexes on the mechanisms of neutralization but not on the antigenic structure.

Besides amino acid substitutions correlated to antigenicity, other positions in VP1 (199, 208, 209 and 214) were found to be changed in several MAR mutants selected with unrelated mAbs. The same residues also change frequently in field isolates, which do not show any antigenic modification with respect to this panel of mAbs (N. J. Knowles, personal communication; Borrego et al., 2000a). These positions occur in the GH-loop of VP1 (Seechurn et al., 1990), which is extremely variable in other picornaviruses. In FMD virus, this region contains the main linear neutralizing site, characterized by a high variability; also, it constitutes an antigenic region for polio- and rhinoviruses (Mateu, 1995; Usherwood & Nash, 1995). It cannot be excluded that for SVDV, this region is antigenically relevant; however, from data reported here, no correlation was found between these amino acid changes and antigenic sites defined by the 11 mAbs. Changes in positions 143 and 167 of VP1 are more difficult to explain as a result of genetic variability or permissivity, as they are found within conserved structural domains among enteroviruses and are not located in exposed loops, but are predicted in the 3D model to be internal (data not shown). A possible role of these positions is still to be determined.

All the neutralizing sites described so far on SVDV, including the new ones reported here, are conformation-dependent, as they are not maintained in any of the denatured capsid proteins. However, some evidence of the involvement of linear epitopes in the immune response of pigs after infection has been reported (Tsuda et al., 1987; Jiménez-Clavero et al., 1998). Furthermore, a wide panel of mAbs raised against linear epitopes of SVDV has been described (Borrego et al., 2000b), but none of them was able to neutralize virus infectivity. Despite the existence of antigenic linear regions on the SVDV capsid, with some of them next to or even overlapping neutralizing sites (Jiménez-Clavero et al., 2000, 2001; Borrego et al., 2000b), all these reports contributed to demonstrate the immunodominance of conformational sites with respect to continuous epitopes. Moreover, the demonstration and characterization of identical neutralizing sites by independent studies and using antigenically different isolates provides further incontrovertible evidence of the importance of those sites.

Our results have improved the understanding of the antigenic structure of SVDV through the description of new neutralizing sites and the characterization of their specificity and stability. These data may have useful applications in diagnostic and epidemiological investigations, as mAbs directed against conserved epitopes, like those identifying antigenic site Ia, provide universal reagents useful for SVDV detection systems, while mAbs against known variable sites readily allow the identification of antigenic variants.

Studies, now in progress, with multiple mutants that are no longer reactive or neutralized by any of the mAbs could allow the identification of potentially new sites not yet identified and improve the knowledge of the relationship between sites already described in order to complete the antigenic map of SVDV. Such mutants will provide an invaluable tool to address and characterize the immune response against selected sets of epitopes, but also to study other phenotypic features, such as pathogenicity, receptor binding, attenuation or virulence, that have been correlated to antigenicity for other picornaviruses.

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