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The N-Terminal Region of the VP1 Protein of Swine Vesicular Disease Virus Contains a Neutralization Site That Arises upon Cell Attachment and Is Involved in Viral Entry

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The N-terminal region of VP1 of swine vesicular disease virus (SVDV) is highly antigenic in swine, despite its internal location in the capsid. Here we show that antibodies to this region can block infection and that allowing the virus to attach to cells increases this blockage significantly. The results indicate that upon binding to the cell, SVDV capsid undergoes a conformational change that is temperature independent and that exposes the N terminus of VP1. This process makes this region accessible to antibodies which block virus entry.

Swine vesicular disease virus (SVDV) is an enterovirus of the *Picornaviridae* family that infects pigs, causing a disease whose symptoms resemble those of foot-and-mouth disease (21). Molecular epidemiological analyses indicate that SVDV recently arose from the human pathogen coxsackievirus B5 (29). Antigenic characterization of SVDV has been carried out by analysis of monoclonal-antibody-resistant (MAR) mutants (14, 22) and by peptide scanning (12).

Analyses of MAR mutants have defined seven neutralization sites, five of which are analogous to the classical 1, 2, 3A, 3B, and 3C sites identified in poliovirus, the type species for the enterovirus group (14), and two of which are in the C termini of structural proteins VP1 and VP3, respectively (22). All neutralization sites identified by means of the MAR mutant analyses are well exposed on the surface of the capsid, as seen in three-dimensional models of the virion (14, 22). However, we have recently found that sera from SVDV-infected pigs recognize other epitopes, not revealed by the MAR mutant analyses, which are located in the capsid but not exposed on its surface (12). Among these antigenic regions, the N terminus of VP1 is of particular interest since, despite being located at the inner side of the capsid shell, it is strongly recognized by antibodies from infected pigs.

According to a widely accepted model, the capsids of picornaviruses, notably poliovirus (3, 7), coxsackievirus (5), and rhinovirus (19), undergo conformational rearrangements upon binding of the virus to the cell receptor. In this process, the capsid transforms into a structurally and antigenically altered form, the A particle, with a lower sedimentation coefficient and increased hydrophobicity and sensitivity to proteases. These A particles are the main form of intracellular virus early after infection (20) and are considered intracellular intermediates that precede viral uncoating (11), although they are also found extracellularly as a result of elution from the receptor after binding. The A particles undergo two specific changes, namely, the externalization of the N terminus of VP1 and the loss of VP4 (3, 7, 15). That this transition is an essential event in the mechanism of infection of many picornaviruses is well illustrated by the fact that antiviral drugs that inhibit a broad range of entero- and rhinoviruses (1, 28) act by stabilizing native virus capsids, thus preventing these conformational changes (10, 17, 23, 27).

The relevance of the immune response to the VP1 N terminus for host protection against poliovirus has been pointed out by in vitro studies of viral neutralization. Synthetic peptides corresponding to this region elicit the production of neutralizing antibodies in mice, rats, and rabbits (4, 18). In addition, this region is immunogenic in humans vaccinated with an attenuated (Sabin) poliovirus vaccine (25), in rabbits inoculated with coxsackievirus A9 (24), and in SVDV-infected pigs (12).

In light of these previous results, we investigated the presence of neutralization sites in the N terminus of SVDV VP1 and the role of this region during infection. To this end, we synthesized the peptide VP1 N-ter (GPPGGVTEGIIARVAD TVGS), spanning the 20 N-terminal residues of the VP1 capsid protein of the SVDV SPA/1/'93 isolate (GenBank accession no. AF039166). Antibodies to this synthetic peptide were produced by immunization of rabbits with two consecutive subcutaneous inoculations of keyhole limpet hemocyanin-coupled peptide conjugate (200 μg each) at 4-week intervals, using QuilA (*Quillaja saponaria* saponing; Superfos Biosector a/s; 0.5% final concentration) as an adjuvant. This antiserum recognized the peptide in an enzyme-linked immunosorbent assay performed as previously described (13) (data not shown). To determine the ability of these VP1 N-ter antibodies to interfere with SVDV infection, we carried out an in vitro neutralization assay based on a previously described protocol (8). Briefly, duplicate 100-PFU inocula of SVDV (SPA/1/'93 isolate) were incubated at 37°C for 30 min with dilutions of the antiserum in 96-well plates. IB-RS-2 cells (a swine kidney cell line; kindly provided by C. Gomez-Tejedor, CISA-INIA, Valdeolmos, Spain [a description of the history of this cell line is found in reference (6)]) were added (2×10^4/well), and the plates were further incubated at 37°C for 18 to 20 h. Noninfected cells, which remained attached to the wells, were formalin fixed and stained with crystal violet. To determine the level of cell sur-

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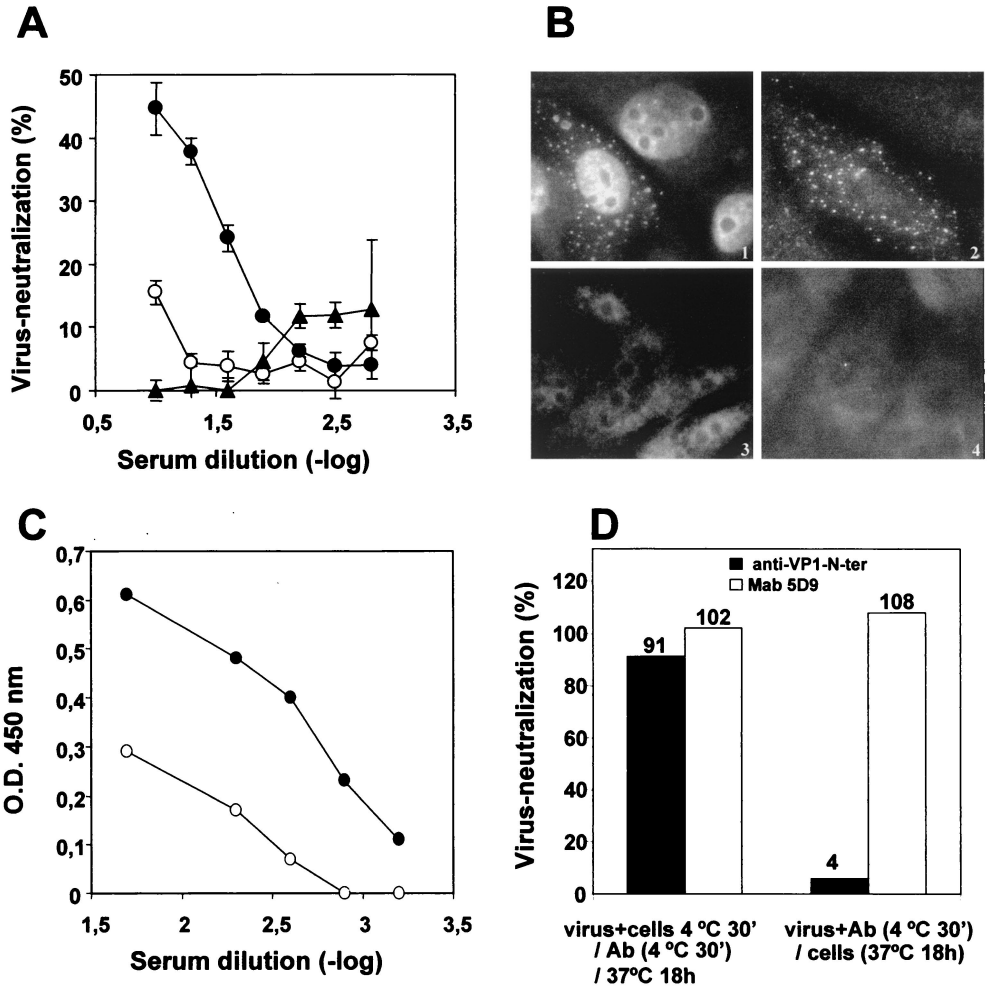

FIG. 1. (A) Virus neutralization titration of the antiserum to VP1 N-ter, using the SPA/1/'93 (●) and UKG/27/'72 (▲) isolates of SVDV. Open circles represent the titration of preimmune serum using SPA/1/'93 SVDV. Each point represents the mean of duplicate determinations. (B) Immunofluorescence staining of SVDV bound to the cell membrane. SVDV particles were adsorbed to IB-RS-2 cells for 30 min at 4°C, washed, and fixed with PFA. Bound viral particles were stained with VP1 N-ter antiserum (panel 1) or with MAb 5D9, which is specific for a surface epitope of the virus (panel 2). The preimmune anti-VP1 N-ter serum (panel 3) and an irrelevant MAb (panel 4) were used as negative controls. Viral particles were stained with a Texas red-conjugated secondary antibody. (C) Binding of the VP1 N-ter antiserum (●) or preimmune serum (○) to SVDV adsorbed to IB-RS-2 cells. Virus was incubated with cells at 4°C and fixed with PFA. O.D., optical density. (D) Neutralization of SVDV attached to cells. Virus was incubated with the VP1 N-ter antibody at 4°C for 30 min, after (left bars) or before (right bars) adsorption to IB-RS-2 cells at 4°C. Cells were then incubated at 37°C to allow viral infection. Virus neutralization was determined as described in the text.

vival, the dye was eluted from the cells by adding 200 μl of methanol/well and the absorbance of each well at 595 nm was measured. The average optical density of uninfected-cell controls represented 0% cytopathic effect (CPE), and that of cells infected in the absence of antibodies was considered 100% CPE. As shown in Fig. 1A, the antiserum to VP1 N-ter specifically neutralized the infection produced by the SVDV SPA/1/'93 isolate with a classical sigmoidal titration curve, reaching 45% CPE inhibition. This virus neutralization titer was similar to that described for antibodies against the poliovirus VP1 N terminus (4, 18). The SVDV UKG/27/'72 isolate, differing from SVDV SPA/1/'93 isolate in 6 amino acid residues of the VP1 N-terminal sequence (26), was not neutralized. These results suggested that the neutralizing epitope is probably located in the variable region of the VP1 N terminus between amino acid positions 5 and 18.

The above results indicated that under these conditions the N terminus of VP1 is accessible to antibodies; thus, it must be exposed on the capsid. However, about 50% of the virus pool was still able to infect the cells at saturating serum antibody concentrations.

As noted above, it has been shown that enterovirus-receptor interaction triggers the structural changes leading to the externalization of the VP1 N terminus. Accordingly, the binding of anti-VP1 N-ter antibodies to the virus could be increased by allowing the virus to interact with the cells before adding the antiserum. This hypothesis was analyzed by immunofluorescence microscopy, using IB-RS-2 cells incubated with SVDV (10 PFU/cell) for 30 min at 4°C and fixed with 4% paraformaldehyde (PFA). At this temperature, the virus-receptor interaction occurs but internalization of the virion is prevented. Cultures were incubated with VP1 N-ter antiserum (Fig. 1B,

panel 1) or with monoclonal antibody (MAb) 5D9, which is specific for a known viral capsid epitope (22) (Fig. 1B, panel 2). Viral particles were stained with a Texas red-conjugated secondary antibody. Both antibodies, but neither the preimmune antiserum nor an irrelevant MAb, bound to the viral particles attached to the cell surface. Apart from specific binding to cell-attached virus, we found some nuclear staining with VP1 N-ter antiserum and, to a lesser extent, with the preimmune serum. This binding is probably due to nonspecific interactions, since under the conditions of this experiment the presence of viral proteins inside the cells was not expected, as immunofluorescence staining with MAb 5D9 clearly showed (Fig. 1B, panel 2). This result indicated that the N terminus of VP1 of the attached virus particles was accessible. This result was confirmed by ELISA (Fig. 1C). SVDV particles (10^6 PFU/well) were incubated for 15 min at 4°C with IB-RS-2 cells grown in 96-well plates. After being washed to remove unbound virus, the cells were fixed in 4% PFA and incubated with the anti-VP1 N-ter serum. The antibodies bound to cell-adsorbed virus were revealed by using an anti-rabbit immunoglobulin coupled to horseradish peroxidase. As shown in Fig. 1C, the anti-VP1 N-ter serum efficiently bound to SVDV adsorbed to cells.

The degree of virus neutralization produced by the VP1 N-ter antibodies was then determined under conditions in which the antipeptide antibodies were bound to SVDV that had been adsorbed to cells at 4°C. SVDV (4×10^3 PFU/well) was adsorbed to IB-RS-2 cell monolayers in 96-well plates for 30 min at 4°C. After being washed with Dulbecco's modified Eagle medium to eliminate unbound virus, the cells were incubated with anti-VP1 N-ter (1:20) for 30 min at 4°C. The cells were washed with Dulbecco's modified Eagle medium and then incubated for 18 to 20 h at 37°C, and the CPE was determined as described above. As shown in Fig. 1D, the level of neutralization achieved by the VP1 N-ter antibody was 91%, which is a magnitude similar to that resulting with the neutralizing MAb 5D9, which is specific for a surface-exposed epitope in the C-terminal region of VP3 (22). It could be argued that the externalization of the VP1 N-ter epitope was an effect of the incubation at 4°C and not a result of the binding to the cells. To eliminate this possibility, the virus was incubated with either the anti-VP1 N-ter serum or MAb 5D9 for 30 min at 4°C and then added to the cells and further incubated at 37°C. In this experiment, the neutralization caused by MAb 5D9 remained at the level seen under previous conditions (100%), but the VP1 N-ter antibody did not neutralize the infection, indicating that binding of the virus to the cells was required for VP1 externalization. In addition, when the in vitro neutralization test (Fig. 1A) was performed in the presence of chloroquine, a drug that impairs the endolysosomic pathway, at a concentration (20 μM) that did not affect SVDV infection, the anti-VP1 N-ter serum blocked 100% of infection (data not shown). This result further supports the hypothesis that the interaction of VP1 N-ter antibodies with the SVDV VP1 N terminus is greatly facilitated by treatments that inhibit endocytosis, probably increasing the period of interaction of the virus with specific receptors on the surface of the cells before its internalization.

Taken together, these results indicate that the neutralization epitope located at the N terminus of SVDV VP1 becomes externalized when the virus attaches to the cells, allowing the

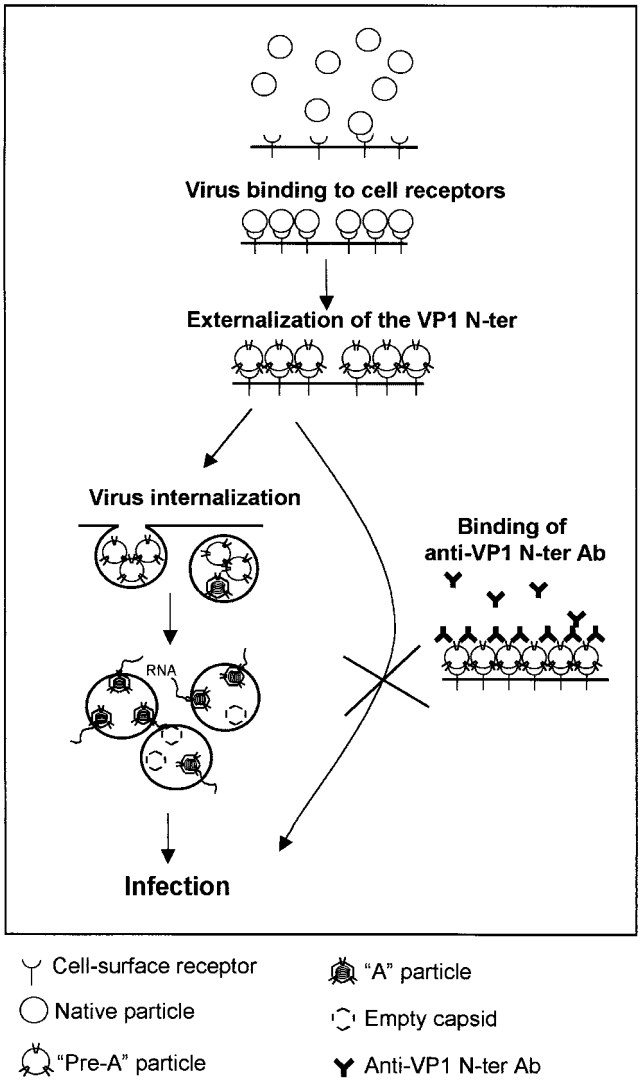

FIG. 2. Framework for the interaction of SVDV with cells, summarizing the data presented in this article. Upon attachment, native virions transform to pre-A particles, in which the VP1 N terminus is externalized. The viral particles become A particles, which are internalized and uncoated. The VP1 N-ter antiserum binds to the virus attached to the cells, but not to native particles or to internalized virus. Therefore, inhibition of the infection by the anti-N-ter serum suggests that internalization of the pre-A particles is prevented after the binding of the antibody to the N terminus of VP1.

binding of antibodies to this site and thereby resulting in a blockage of the infection. In poliovirus, the normally internal N terminus of VP1 is known to be exposed on the capsid surface in at least two different circumstances: during a reversible change occurring at physiological temperatures in solution (virus "breathing"), and as the result of an irreversible process triggered by binding of the virus to the cell receptor, leading to the production of A particles. Both processes take place at 37°C and are inhibited at 4°C (7, 18). Furthermore, at least 15 min of incubation at 37°C is required for detection of significant conversion to A particles (7, 9, 16). The results that we observed in this work suggest that in SVDV there is an intermediate stage in the process of viral entry, involving what we

have named pre-A particles. These particles can be found on the cell surface as soon as 15 min after the virus is added to the cell cultures. In addition, the transition of the native form to the pre-A particle can take place at 4°C, provided that the virus has interacted with the cellular receptor. Thus, the externalization of the VP1 N terminus of SVDV could take place before the transition to A particles seen in poliovirus. This model is in accordance with recent data showing that in poliovirus, the externalization of the VP1 N terminus and the loss of VP4 are two independent events that can be uncoupled (2). These observations were made in studies using poliovirus empty capsids, and the authors showed that in these particles the N terminus of VP1 can be externalized under conditions in which VP4 remains inside the virion, allowing an efficient attachment to cells in a receptor-dependent mechanism.

To illustrate the observations made in the present work, we propose the mechanism shown in Fig. 2 for the events mediating the entry of SVDV into the cell. The interaction of the virus with the cell receptor triggers the externalization of the VP1 N-ter epitope, and probably the opening of the capsid pores; thus, all attached viruses become pre-A particles. In normal infections, these pre-A particles become A particles (similar to the A particles described for poliovirus), which are intracellular intermediates that precede viral uncoating. The interaction of the VP1 N-ter antibody with the virus attached to the cell surface (pre-A particles) results in complete neutralization, suggesting that the binding of VP1 N-ter antibody to the virus blocks its internalization or another event following virus attachment.

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