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Interaction of Classical Swine Fever Virus with Membrane-Associated Heparan Sulfate: Role for Virus Replication In Vivo and Virulence

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Passage of native classical swine fever virus (CSFV) in cultured swine kidney cells (SK6 cells) selects virus variants that attach to the surface of cells by interaction with membrane-associated heparan sulfate (HS). A Ser-to-Arg change in the C terminus of envelope glycoprotein E^{rns} (amino acid 476 in the open reading frame of CSFV) is responsible for selection of these HS-binding virus variants (M. M. Hulst, H. G. P. van Gennip, and R. J. M. Moormann, J. Virol. 74:9553–9561, 2000). In this investigation we studied the role of binding of CSFV to HS in vivo. Using reverse genetics, an HS-independent recombinant virus (S-ST virus) with Ser^{476} and an HS-dependent recombinant virus (S-RT virus) with Arg^{476} were constructed. Animal experiments indicated that this adaptive Ser-to-Arg mutation had no effect on the virulence of CSFV. Analysis of viruses reisolated from pigs infected with these recombinant viruses indicated that replication in vivo introduced no mutations in the genes of the envelope proteins E^{rns}, E1, and E2. However, the blood of one of the three pigs infected with the S-RT virus contained also a low level of virus particles that, when grown under a methylcellulose overlay, produced relative large plaques, characteristic of an HS-independent virus. Sequence analysis of such a large-plaque phenotype showed that Arg^{476} was mutated back to Ser^{476}. Removal of HS from the cell surface and addition of heparin to the medium inhibited infection of cultured (SK6) and primary swine kidney cells with S-ST virus reisolated from pigs by about 70% whereas infection with the administered S-ST recombinant virus produced in SK6 cells was not affected. Furthermore, E^{rns} S-ST protein, produced in insect cells, could bind to immobilized heparin and to HS chains on the surface of SK6 cells. These results indicated that S-ST virus generated in pigs is able to infect cells by an HS-dependent mechanism. Binding of concanavalin A (ConA) to virus particles stimulated the infection of SK6 cells with S-ST virus produced in these cells by 12-fold; in contrast, ConA stimulated infection with S-ST virus generated in pigs no more than 3-fold. This suggests that the surface properties of S-ST virus reisolated from pigs are distinct from those of S-ST virus produced in cell culture. We postulate that due to these surface properties, in vivo-generated CSFV is able to infect cells by an HS-dependent mechanism. Infection studies with the HS-dependent S-RT virus, however, indicated that interaction with HS did not mediate infection of lung macrophages, indicating that alternative receptors are also involved in the attachment of CSFV to cells.

Classical swine fever is a highly contagious and sometimes fatal viral disease of pigs. The causative agent, classical swine fever virus (CSFV), is a member of the *Pestivirus* genus within the family *Flaviviridae* (12). The two other members of this genus are bovine viral diarrhea virus and border disease virus. CSFV has been found to be infectious only for pigs. Bovine viral diarrhea virus and border disease virus can infect both ruminants and pigs (6).

Pestiviruses are small, enveloped, plus-strand RNA viruses (33). The RNA genome is approximately 12.5 kb (3, 7, 30, 34) and contains a single large open reading frame (ORF) (3, 8, 30, 34). This ORF is translated into a polyprotein which is further processed into mature proteins by viral and host cell proteases (38). The surface structure of pestivirus virions is composed of three glycoproteins, E^{rns}, E1, and E2 (46). E2 is present as a homodimer and as an E2-E1 heterodimer (46, 51). The amino acid C terminus of E2 (and probably of E1 as well) functions as a membrane-spanning domain (14) and anchors these E2-E1 and E2-E2 dimers in the viral lipid membrane. Association of E^{rns} homodimers with the virion is not accomplished by a membrane-spanning domain and is tenuous (11). The mechanism of E^{rns} association with virions is currently unknown. All three envelope proteins contain N-linked glycosyl groups (38, 46, 51). Compared to E1 and E2, E^{rns} is glycosylated to a higher extend. N-linked glycosyl residues account for about half of the mass of an E^{rns} homodimer (38, 54). A considerable part of E^{rns} produced in infected cells is secreted into the extracellular environment and circulates in the body fluids of infected animals (38). The unexpected finding that E^{rns} possesses RNase activity (15, 42) led to several interesting studies regarding the function of E^{rns} in the life cycle of pestiviruses. In vitro and in vivo studies indicated that E^{rns} (and its enzyme activity) plays a role in regulation of RNA synthesis in infected cells (17) and in weakening the immune defense of the host, early in infection (4, 31).

Infection studies with cultured swine kidney cells showed that pestivirus infection is at least a two-step process (16). First, interaction of E^{rns} with the cell surface mediates the attachment of virions to the cell surface. Second, interaction of E2 with a second, probably more specific surface molecule is needed to mediate penetration of cells (16, 55). Although

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several surface proteins were identified as putative receptors for pestiviruses, none of these proteins have been characterized thoroughly. Using anti-idiotypic antibodies, a 50-kDa cell surface protein was identified as an E2-specific receptor (55). Also the low-density lipoprotein receptor (1) and a 60-kDa actin-binding surface protein (40) were designated as possible receptors. Recently, it was demonstrated that in vitro cultivation of CSFV in swine kidney cells selects virus variants that attach to the cell surface of cultured cells by interaction with the membrane-associated glycosaminoglycan (GAG) heparan sulfate (HS) (18). Using reverse genetics, it was shown that replacement of a neutral serine residue by a positively charged arginine residue in the C terminus of E^{rns} (position 476 in the ORF) changes an HS-independent CSFV to a virus that uses HS as an E^{rns} receptor (18). These data clearly showed that interaction of virus bound E^{rns} with HS immobilizes CSFV at the cell surface of cultured swine kidney cells.

GAGs, like HS, are unbranched polysaccharide chains composed of repeated disaccharide sequences. Multiple chains are covalently linked to a protein core, forming a network of chains (proteoglycans) exposed on the surface of virtually all types of cells (21, 25). Sulfate groups are N and O linked to the sugar residues and give the GAG chains a net negative charge (9, 13). The positions at which sulfate groups and other moieties, like N-linked acetyl groups, are linked to the sugar chain determine the large diversity of HS chains found in nature (21, 25, 44). Binding of proteins to HS is not dependent only on electrostatic forces between positively charged amino acids and negatively charged clusters of the HS chains; because of this diversity, it can be quite specific (25, 43, 44). A wide variety of pathogens (37), including many viruses (reference 5 and references herein), bind to HS.

For foot-and mouth disease virus (FMDV) and Sindbis virus (SV), for which binding to HS has been reported, animal experiments showed that HS-dependent virus variants selected in cell culture are less virulent than their HS-independent counterparts (27, 39). In this investigation we studied the role of HS binding for CSFV in vivo. Using reverse genetics, an HS-independent virus with Ser^{476} and an HS-dependent virus with an Arg^{476} in the C terminus of E^{rns} were constructed from a virulent strain of CSFV. Infection of pigs with these recombinant viruses indicated that this adaptive Ser-to-Arg mutation did not reduce the virulence of CSFV. Furthermore, we showed that virus reisolated from pigs infected with the HS-independent recombinant virus is able to infect cultured and primary swine kidney cells by an HS-dependent mechanism. Sequence analysis showed that replication in vivo introduced no mutations in the E^{rns}, E1, and E2 genes of this virus. Our results indicate that the surface properties of CSFV generated in pigs, carrying Ser^{476} in the C terminus of E^{rns}, are distinct from those of genetically identical virus produced in cell culture.

MATERIALS AND METHODS

Cells and viruses. Swine kidney cell line SK6 (24), was maintained as described previously (16). Porcine lung macrophages were isolated from lungs of 6-week-old specific-pathogen-free piglets as described recently (52). Primary swine kidney cells were prepared using kidneys of 6-week-old specific-pathogen-free pigs as described previously (2). After preparation, these cells were grown as monolayer for 4 days in Earle's minimal essential medium (EMEM) containing 10% fetal bovine serum FBS and antibiotics before being treated with trypsin, suspended in Eagle basal medium containing 5% FBS and antibiotics (SK6 tissue culture medium), and divided among 2-cm^2 tissue culture wells (M24; Costar). FBS and cells were free of pestivirus, and the FBS was free of pestivirus antibody. Blood of a pig infected with the virulent CSFV field isolate Brescia (456610) was used as a control in the animal experiment (49). Isolation of the HS-independent Brescia virus clone Bp2, and its once-extra-passaged-in SK6-cells HS-dependent counterpart, Bp3, have been described recently (18). The recently constructed recombinant virus flc.1.1.1.E^{rns}(R-RI) (18), derived from Brescia virus clone C1.1.1 (49), was used in concanavalin A (ConA) assays.

Isolation and sequencing of cDNA of virus clone Bp3. Cytoplasmic RNA isolated from SK6 cells infected with Brescia virus clone Bp3 (18) was used as template in reverse transcription-PCR (RT-PCR) to generate overlapping cDNA fragments covering the complete RNA genome. RT reactions were performed with the Superscript II enzyme (Gibco-BRL), and PCRs were performed with the high-fidelity PCR system (Boerhringer Mannheim). Primers used for amplification of these cDNA fragments were derived from the published sequence of CSFV strain Brescia C1.1.1 (34). DNA fragments were cloned in pGEM-Teasy and sequenced as described previously (17). The exact sequences of the 5′ and 3′ termini of the RNA genome of clone Bp3 were determined in the same manner as described for the genome of clone C1.1.1 and CSFV strain C (35). The entire genomic consensus sequence of virus clone Bp3 was determined by sequencing three cDNA fragments, obtained from independent PCRs.

Construction, generation, and characterization of recombinant viruses. A full-length DNA copy of virus clone Bp3 was constructed by joining cDNA fragments, isolated from pGem-Teasy plasmids, in the low-copy-number plasmid pOK12. Construction was performed in the same manner as described for the full-length cDNA of CSFV strain C (35). The junction between the T7 RNA polymerase promoter sequence and the 5′-terminal nucleotide of the Bp3 cDNA and the junction between the 3′-terminal nucleotide of Bp3 cDNA and the vector were similar to those described for the full-length copy of strain C in pOK12 (35). Digestion of this full-length cDNA in pOK12 with *Srf*I generates a template that after transcription with T7 RNA polymerase produces the exact 3′ terminus of the RNA genome of virus clone Bp3. This full-length cDNA was named pflc.CoBrB. In the text and figures of this paper, this full-length cDNA, and the virus derived from it, are named pflc.CoBrB.E^{rns}(S-RT) and CoBrB.E^{rns}(S-RT) virus, respectively. To construct a full-length recombinant virus with an E^{rns} S-ST gene, the *Cla*I-*Ngo*MIV cDNA fragment generated previously from virus clone Bp2 [covering the C-terminal part of N^{pro}, the capsid protein (C), E^{rns}, and E1], was used to replace the *Cla*I-*Ngo*MIV cDNA fragment of pflc.CoBrB.E^{rns}(S-RT) to give pflc.CoBrB.E^{rns}(S-ST). Sequence analysis of pflc.CoBrB.E^{rns}(S-ST) showed that the nucleotide mutation that results in an Arg-to-Ser change at position 476 in the polyprotein of CSFV (18) is the only difference between the *Cla*I-*Ngo*MIV region of pflc.CoBrB.E^{rns}(S-ST) and that of pflc.CoBrB.E^{rns}(S-RT). To generate the recombinant viruses CoBrB.E^{rns}(S-ST) and CoBrB.E^{rns}(S-RT), *Srf*I-linearized DNA (250 ng) of full-length plasmids pflc.CoBrB.E^{rns}(S-ST) and pflc.CoBrB.E^{rns}(S-RT) was transfected to SK6.T7a5 cells as described recently (47). Two days after transfection, the medium was harvested and stored at -70°C and cells were immunostained with monoclonal antibody (MAb3) directed against E2 (50). Then 500 μl of the medium collected from wells in which E2 expression was detected (virus passage 1 [p1]) was used to infect confluent monolayers of SK6 cells, grown in 75-cm^2 tissue culture flasks. After 4 days of growth, the cells and medium were frozen-thawed twice or medium and cells (in fresh medium) were frozen-thawed twice separately, to prepare p2 virus stocks.

Animal experiments. CSFV strain Brescia 456610 (control virus) and recombinant virus stocks (p2) were diluted in SK6 tissue culture medium to a concentration of 10^5 50% tissue culture infective doses per ml (as determined by titer determination on SK6 cells). Groups of three 6-week-old, specific-pathogen-free pigs were infected by spraying 1 ml of this virus dilution in each nostril on day 0. All animals were observed daily for signs of disease, and body temperatures were measured. Fever is defined here as body temperature of >40°C on at least two consecutive days. EDTA-treated blood samples were obtained on days 0,2,4,7,9,11,14,16,18,21, and 23. These blood samples were used for isolation of virus from leukocytes (WBC) (see below) and for monitoring of the concentrations of thrombocytes and WBC. Thrombocytes and WBC were counted as described previously (10). Serum samples were taken on days 0, 7, 14, 21, and 23 and tested in an enzyme-linked immunosorbent assay (ELISA) that specifically detects antibodies directed against envelope protein E2 (Ceditest-Ab kit). The pigs were killed when moribund or slaughtered at the end of the trial. Tissue samples of the spleen, kidney, tonsil, and ileum were collected postmortem frozen in liquid nitrogen, and stored at -70°C. Duplicate cryostat sections of these tissue samples were tested for viral antigen using a direct immunofluorescence technique (10).

Virus isolation from clinical samples. WBC were isolated from EDTA-treated blood as described recently (10). Isolated WBC were resuspended in the same volume of medium (Eagle basal medium containing 10% FBS and antibiotics) as the volume of blood that was used for isolation. WBC suspensions were stored at −70°C. From tissues, 10% (wt/vol) suspensions in EMEM containing 5% FBS and antibiotics were prepared as described previously (10). These suspensions were stored at −70°C. EDTA-treated blood was frozen at −70°C without further treatment. All WBC, tissue, and blood samples, frozen once at −70°C were thawed, divided into aliquots, and stored again at −70°C. In all tests, these twice-frozen-thawed virus samples were used. A 100-μl volume of EDTA blood from a pig infected with CoBrB.E^{rns}(S-ST) was used to infect SK6 cells grown in a 25-cm^2 tissue culture flask. After 90 min of infection, the virus was removed and the cells were washed twice and supplied with fresh medium. After 4 days of growth, the cells and medium were frozen-thawed twice and clarified to prepare a p1 virus stock.

Genetic analysis of in vivo-generated virus. To determine the sequence of the E^{rns}, E1, and E2 genes of virus in the blood of infected pigs, SK6 cells grown in 2-cm^2 tissue culture wells were infected with 50 μl of EDTA-treated blood, diluted in 0.5 ml of EMEM without FBS and antibiotics, for 90 min at 37°C. The virus was removed, and the cells were washed twice with EMEM and supplied with EMEM supplemented with 10% FBS, antibiotics, and 1% methylcellulose (overlay medium). After 2 days of growth, cytoplasmic RNA was extracted from these monolayers and used to determine the sequence of the E^{rns}, E1, and E2 genes (17).

Chemicals, enzymes, and recombinant proteins. Heparin (195 U/mg from porcine intestinal mucosa), ConA, and the enzyme heparinase I (EC 4.2.2.7; 716 mIU/mg [430 Sigma units/mg]) were obtained from Sigma, St. Louis, Mo. Lyophilized enzyme was dissolved in storage buffer and stored in aliquots at −70°C as described previously (18). Heparin and ConA were dissolved in EMEM without FBS and antibiotics and stored for no longer than 2 weeks at 4°C. Recombinant baculoviruses, in which the E^{rns} genes of CSFV strain Brescia (encoding amino acids 268 to 494 of the CSFV polyprotein [34]) clone Bp2 (E^{rns} S-ST) or Bp3 (E^{rns} S-RT) were inserted in the polyhedrin locus, were generated using the Bac-to Bac expression system (Gibco-BRL). Both versions of E^{rns}, expressed by these recombinant baculoviruses, were purified from insect cells by immunoaffinity (15). Purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions and tested for RNase activity as described previously (17).

Inhibition experiments. For the plaque assay, confluent monolayers of SK6 cells or primary swine kidney cells, grown in 2-cm^2 tissue culture wells, were washed twice with EMEM without FBS and antibiotics (EMEM). The cells were preincubated at 37°C for 30 min with 100 μl of EMEM plus different concentrations of heparin. A 100-μl volume of a dilution of a virus stock in EMEM was added to the wells, mixed, and incubated as described above. When the virus solution was added, the concentration of heparin in the wells was diluted twofold. The concentration used in the text and figures hereafter corresponds to this diluted concentration (the concentration at which inhibition actually is measured). After 30 min, the virus was removed and the wells were washed twice with 0.5 ml of EMEM and supplied with overlay medium (see above). Cells were grown for 18 h at 37°C, and infectious centers (hereafter referred to as plaques) were detected by immune staining with E2-specific MAb3. Positive plaques in a well were counted with a microscope. When more than about 250 plaques per well were present, a minimum of 100 plaques in a fixed area (at a magnification of ×40) were counted to calculate the total number of plaques in these wells. The percent inhibition was calculated using the formula $100 \times [1 - (e/c)]$, where c is the average number of plaques in duplicate or triplicate wells to which no heparin was added (control well) and e is the average number of plaques in duplicate or triplicate wells to which heparin was added. Percent infection compared to control wells was calculated using the formula $100 \times (e/c)$. For all virus samples, the percent inhibition was determined at two different multiplicities of infection. For all samples, no significantly different percentages were measured when different amounts of viruses were tested for inhibition by heparin. Therefore, percentages measured at the highest multiplicity of infection are presented. Relative plaque sizes of viruses were scored in wells to which no heparin was added after 48 h of growth under methylcellulose.

Treatment of cells with heparinase I. Confluent monolayers of SK6 cells or primary swine kidney cells, grown in 2-cm^2 tissue culture wells, were washed twice with binding buffer (phosphate-buffered saline containing 0.2% bovine serum albumin, 0.5 mM $CaCl_2$, and 0.5 mM $MgCl_2$). The cells were incubated with 200 μl of binding buffer containing an appropriate concentration of heparinase I. After incubation for 2 h at room temperature with gentle shaking, the enzyme solutions were removed and the cells were washed twice with 0.5 ml of binding buffer. The cells were infected with 200 μl of an appropriate virus dilution in binding buffer. After 30 min of infection at 37°C, the virus was removed and the cells were washed twice with binding buffer, supplied with overlay medium, and further treated as described for a plaque assay. The percent infection compared to control wells or the percent inhibition of infection was calculated by using the same formulas as described above. All percentages are calculated from the average number of plaques in duplicate or triplicate wells.

Macrophage plaque assay. Inhibition of infection of alvolear macrophages with CSFV by heparin and reduction of virus infection of these cells after heparinase I digestion was measured by a modified plaque assay. Macrophages were suspended in RPMI medium containing 5% FBS and antibiotics, transferred to 2-cm^2 tissue culture wells (approximately 10^6 cells/well), and incubated for 2 h at 37°C to allow attachment of cells. For heparin inhibition assays, washing, preincubation, and infection of cells were performed in a similar fashion to that described for SK6 and primary swine kidney cells except that RPMI medium was used instead of EMEM. Heparinase I treatment of cells and infection of these treated cells were performed in the same buffer as that used for SK6 and primary swine kidney cells. For both assays, after infection the virus was removed and the cells were washed twice with RPMI medium without FBS or antibiotics. After incubation of cells for 1 h at 37°C in RPMI medium containing 5% FBS, uninfected SK6 cells, suspended in RPMI medium containing 5% FBS, were added. The cells were incubated for 2 h at 37°C to allow attachment of SK6 cells. Subsequently, the medium was removed and the wells were supplied with overlay medium and further treated as described for a plaque assay. In this manner, a monolayer of SK6 cells on top of the macrophages prevented the loss of macrophages during fixation and staining procedures.

ConA inhibition-stimulation assay. Viruses were diluted in EMEM containing different concentrations of ConA and incubated for 30 min at 37°C. Confluent monolayers of SK6 cells grown in 2-cm^2 tissue culture wells were washed twice with EMEM and infected with these preincubated mixtures for 30 min at 37°C. Virus-ConA mixtured were removed from the cells, and the cells were washed twice with 0.5 ml of EMEM and incubated for 30 min with 0.5 ml of EMEM containing 100 mM methyl-α-D-mannopyranoside (MMP) at 37°C. After removal of the MMP solution, the cells were washed once with EMEM and supplied with overlay medium. After 24 h of growth, the cells were further treated as described for a plaque assay.

Binding of E^{rns} to immobilized heparin. Prepacked heparin columns (1 ml; Hitrap-Sepharose [Pharmacia]) were preeluted with 5 ml of 10 mM phosphate buffer (pH 7.0). Approximately 50 μg of E^{rns}, purified from insect cells, was diluted to 1 ml with 10 mM phosphate buffer (pH 7.0) and loaded on Hitrap columns at a flow rate of 1 ml per min using a peristaltic pump. Bound material was eluted at a flow rate of 1 ml per min by increasing the NaCl concentration stepwise. Fractions were collected (1 ml) and assayed for E^{rns} in an E^{rns}-specific ELISA as described previously (17). The concentration of NaCl in the fractions was determined by measuring the osmolarity with an osmometer (model 3D3; Advanced Instruments Inc.). Hitrap columns of the same lot number were used for both experiments.

Detection of E^{rns} at the surface of SK6 cells. Surface detection of E^{rns} was performed as described previously (16). Briefly, confluent monolayers of SK6 cells grown in 1-cm^2 tissue culture slides (Nunc) were washed twice with EMEM. Purified E^{rns} was diluted to a concentration of 50 μg/ml in EMEM containing 200 μg heparin per ml or no heparin. Solutions were added to the wells, and cells were incubated for 2 h at 37°C. Solutions were removed from the cells, and the wells were washed three times with 0.3 ml of EMEM with or without 200 μg of heparin per ml. The cells were fixed with acetone and stained with an appropriate dilution of an E^{rns}-specific horseradish peroxidase-conjugated polyclonal antibody 716 (17).

RESULTS

Construction and characterization of recombinant viruses. Recently we demonstrated that only a few passages of native CSFV (strain Brescia) in cultured SK6 cells selected virus variants that bind to membrane-associated HS (18). Genetic analysis of HS-independent virus clone Bp2 and its once-extra-passaged HS-dependent counterpart Bp3 showed that a Ser-to-Arg change in the C terminus of E^{rns} (amino acid 476 in the polyprotein) is responsible for altering an HS-independent virus to a virus that uses HS as an E^{rns} receptor. This was further proven by replacement of the E^{rns} gene of an infectious DNA copy of Brescia virus clone C1.1.1 with that of clones Bp2 and

TABLE 1. Infection of pigs with recombinant viruses[a]

Virus	Pig no.	Time (days p.i.) of first observation of[b]:			Time (days p.i.) of death[c]	Time (days p.i.) of first detection of virus in[d]:		Viral detection in organs[e]
		Fever	Leukopenia	Thrombocytopenia		WBC	Blood	
CoBrB(S-RT)	4060	3	2	4	22	4	4	+
	4061	3	2	7	14	7	4	+
	4062	4	4	7	S	7	7	+
CoBrB(S-ST)	4069	2	4	4	14	4	4	+
	4070	2	4	4	14	4	4	+
	4071	2	2	2	14	4	4	+
Control	4072	2	4	4	14	4	4	+
	4073	2	4	4	14	4	4	+
	4074	3	7	7	S	4	4	+

[a] Groups of three specific-pathogen-free pigs were infected intranasally with 10^5 50% tissue culture infective doses of recombinant viruses or control virus (Brescia 456610) per nostril on day 0. All animals were observed daily for signs of disease, and body temperatures were measured.
[b] Fever (>40 °C), leukopenia, and thrombocytopenia were regarded as signs of disease. EDTA-treated blood samples were taken, and WBC and thrombocytes were counted. Less than 8×10^9 WBC per ml and 2×10^{11} thrombocytes per ml was regarded as indicating leukopenia and thrombocytopenia, respectively.
[c] Pigs 4062 and 4074 survived (S) and were slaughtered in day 23 p.i.
[d] WBC and EDTA-treated blood were used to infect SK6 cells. After 2 days of growth, virus infected cells were detected by immunostaining.
[e] After slaughter, viral antigens in spleen, tonsil, kidney, and ileum tissue were detected by direct immunofluorescence.

Bp3 (18). C1.1.1 was cloned after 32 successive passages in cultured swine kidney cells (49). Because C1.1.1 is avirulent in pigs (49), these recombinant viruses are not suitable for use in studies of the role of HS binding in vivo with regard to virulence. Therefore, an infectious DNA copy of Brescia virus that was not extensively passaged in SK6 cells was constructed. RNA isolated from SK6 cells infected with Brescia clone Bp3 was used as template in standard RT-PCRs. Generated cDNA fragments covering the complete genome of CSFV were cloned, sequenced, and used to construct a full-length DNA copy of the HS-dependent virus clone Bp3, named pflc.Co BrB.E^{rns}(S-RT) (H.G.P. van Gennip et al., unpublished data). To construct an HS-independent DNA copy, named pflc.Co BrB.E^{rns}(S-ST), a cDNA fragment covering the complete C, E^{rns}, and E1 genes of virus clone Bp2 was used to replace the corresponding cDNA fragment of pflc.CoBrB.E^{rns}(S-RT). The only difference between the Bp2 fragment and the replaced fragment of pflc.CoBrB.E^{rns}(S-RT) is the nucleotide mutation that results in an Arg-to-Ser change in the C terminus of the E^{rns} (amino acid 476 of the polyprotein) Transfection of *Srf*I-linearized vector DNAs into SK6.T7 cells (47) yielded the infectious recombinant viruses CoBrB.E^{rns}(S-RT) and Co BrB.E^{rns}(S-ST). The transfection medium was used to infect SK6 cells in order to produce p2 virus stocks. These virus stocks were tested for inhibition of infection by heparin and for reduction of infection after heparinase I treatment of cells (18). Infection of SK6 cells with CoBrB.E^{rns}(S-RT) was inhibited by 95% by 200 μg of heparin per ml and inhibited by 95% by treatment of cells with 12.5 mIU of heparinase I per ml. In contrast, infection of SK6 cells with CoBrB.E^{rns}(S-ST) was not significantly affected by heparin or by treatment of cells with heparinase I [see also Fig. 2; SK6(S-ST)]. When SK6 cells were grown under a methylcellulose overlay, the diameter of S-ST plaques was two to three times larger than the size of S-RT plaques (18). Furthermore, the titer of p2 virus stocks measured on SK6 cells was about 100-fold lower for CoBrB.E^{rns}(S-ST) than for CoBrB.E^{rns}(S-RT) (4.7 and 6.6 $\log_{10}$ PFU/ml, respectively). These results clearly showed that infection of SK6 cells with CoBrB.E^{rns}(S-RT) occurred by an HS-dependent mechanism and that infection with CoBrB.E^{rns}(S-ST) occurred by an HS-independent mechanism. Moreover, as observed for the HS-independent C1.1.1 recombinant virus [flc.1.1.1.E^{rns}(S-ST)] (18), one additional passage in SK6 cells was needed to convert the HS-independent CoBrB.E^{rns}(S-ST) virus to an HS-dependent variant (results not shown).

Virulence in pigs. Pigs were infected intranasally according to the regime described in Table 1, footnote *a*. Pigs in all three groups rapidly developed fever (>40°C) and symptoms of acute CSF. All pigs became seriously ill, as indicated by lack of appetite, vomiting, dullness, blue ears, and paresis. They all developed leukopenia and thrombocytopenia. The three pigs infected with the S-ST virus seemed to respond slightly faster than the pigs infected with the S-RT virus and one of the pigs (animal 4074) infected with native Brescia (control virus). Pig 4062, infected with the S-RT virus, and pig 4074, infected with the control virus, developed the chronic form of CSF. No antibodies against E2 were detected in serum samples from these two pigs and they stayed viremic until the end of the trial. After slaughter, viral antigen could also be detected in spleen, tonsil, kidney; and ileum samples from these two pigs.

Genetic analysis of in vivo-generated virus. Blood samples, taken from pigs shortly before death, were subjected to titer determination in a plaque assay to determine the plaque size after 2 days of growth under methylcellulose. In wells infected with blood samples from the three pigs infected with Co BrB.E^{rns}(S-ST) (containing about 50 to 100 plaques per 2 cm^2), all plaques were large and similar in size to plaques formed by the CoBrB.E^{rns}(S-ST) virus produced in SK6 cells. In wells infected with blood samples taken from the three pigs infected with CoBrB.E^{rns}(S-RT) virus (containing about 200 to 500 plaques per 2 cm^2), all plaques were small and similar in size to the plaques formed by the CoBrB.E^{rns}(S-RT) virus produced in SK6 cells. The blood samples from pig 4071 (S-ST) and 4061 (S-RT) (both taken on day 14 postinfection [p.i.])

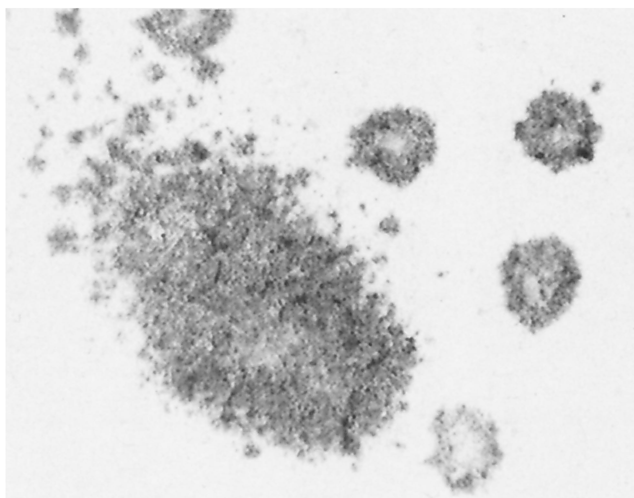


FIG. 1. Detection of a large-plaque phenotype in the blood of pig 4062 infected with CoBrB.E^{rns}(S-RT). Blood from pig 4062 was subjected to titer determination in an SK6 plaque assay in the presence of 200 μg of heparin per ml. After 2 days of growth under methylcellulose, the plaques were stained. The small round plaques are characteristic of an HS-dependent virus (S-RT). The large plaque is similar in size and shape to plaques produced by an HS-independent S-ST virus (18).

that were analyzed above for plaque phenotype were used to infect SK6 cells. After 2 days of growth under methylcellulose, RNA was extracted from these cells (18) and used to determine the sequences of the E^{rns}, E1, and E2 genes. For both reisolated viruses, the E^{rns}, E1, and E2 sequences were identical to the sequences of the viruses administered to the pigs. These results showed that replication of both recombinant viruses in pigs did not induce genetic changes in the envelope proteins. However, when blood samples from pig 4062, taken on days 14 and 22 p.i., were subjected to titer determination in a plaque assay in the presence of 200 μg of heparin per ml, a few plaques with a large phenotype were detected (Fig. 1). No large plaques were detected when other blood samples and tissue samples, collected from pig 4062 or from the two other S-RT pigs, were subjected to titer determination in the presence of heparin. Taking into account that 200 μg of heparin per ml inhibited infection by the S-RT virus by approximately 95%, it was estimated that no more than 1 in 4,000 virus particles in these blood samples produces a large plaque. To analyze this further, one large plaque was scraped from the immunostained monolayer. After the addition of uninfected SK6 cells, RNA was isolated and used as template in an RT-PCR (17). Sequence analysis of the generated cDNA fragment, encoding the C-terminal part of E^{rns} (amino acids 467 to 494), showed that the viral RNA isolated from this plaque codes for a Ser at position 476 in the polyprotein. This indicated that Arg^{476} was mutated to a Ser^{476}. Because of the low concentration of this large-plaque phenotype(s) in these blood samples, we did not attempt to isolate and further characterize this virus variant(s).

Infection of cultured cells with in vivo-generated virus. Recently, we isolated virus clones from blood and organs of pigs infected with CSFV, using limited passage in SK6 cells (18). Without further passage in SK6 cells, all these virus clones (produced in SK6 cells) infected SK6 cells by an HS-independent mechanism (18). In contrast, when these clinical blood and organ samples were directly tested (without passage in cell culture) in plaque assays, infection of SK6 cells was reduced 50% or more by heparin and heparinase I treatment of cells. Furthermore, sequence analysis of cDNA fragments, generated by RT-PCR from viral RNA present in the blood sample that was used for isolation of these virus clones, showed that the E^{rns} consensus sequence established for virus in this blood sample was identical to the E^{rns} sequences of these clones (18). Therefore, we speculated that the ability of in vivo virus to attach to the cell surface by interaction with HS is a result of its specific surface properties. Surface properties can be different from those of virus produced in SK6 cells. To find additional evidence for this hypothesis, organ suspensions, white blood cells (WBC), and blood of pigs infected with both recombinant viruses were subjected to titer determination on SK6 cells in a plaque assay with or without 200 μg of heparin per ml. In addition, blood and WBC samples were tested in a heparinase I assay. In Table 2 the results of these tests are presented for pigs 4071 (S-ST) and 4061 (S-RT). Test results obtained with samples taken from the two other pigs of each group were not significantly different from those for pigs 4071 and 4061. All clinical samples from pig 4071 formed only large plaques and all clinical samples from pig 4061 formed only small plaques on SK6 cells. Infection of SK6 cells with virus samples taken from pig 4061 (S-RT) was almost completely inhibited by 200 μg of heparin per ml. Virus samples from pig 4071 were inhibited 55 to 75% by 200 μg of heparin per ml. Removal of HS from the cell surface inhibited infection with S-ST and S-RT viruses present in blood and WBC samples to the same extent as heparin did. Note that the virus titers in suspensions of organs from pig 4061 infected with the S-RT virus were about 500-fold higher when determined on SK6 cells and 10- to 20-fold higher when determined on macrophages than were the titers in the suspensions of organs from pig 4071 infected with the S-ST virus. In addition, heparin and heparinase I treatment inhibited infection of SK6 cells with blood of pig 4071 and 4061 in

TABLE 2. Characterization of clinical samples

Pig (virus)	Sample[a]	Titer (log_{10} PFU/ml) (SK6/Macr)[b]	% Inhibition by heparin (heparinase)[c]	Plaque size[d]
4071 (S-ST)	Tonsil	6.5/5.7	65	Large
	Kidney	6.3/5.7	55	Large
	WBC	4.0/ND[e]	70 (75)	Large
	Blood	7.5/6.6	75 (80)	Large
4061 (S-RT)	Tonsil	9.3/6.9	95	Small
	Kidney	9.2/6.8	95	Small
	WBC	5.8/ND	95 (95)	Small
	Blood	7.6/6.1	90 (95)	Small

[a] Tonsil and kidney samples were prepared postmortem at 14 days p.i. EDTA-treated blood, taken on day 12 p.i., was tested and used to isolate WBC fractions.

[b] Titers were measured in a plaque assay using SK6 cells (SK6) or lung macrophages (Macr).

[c] Percent inhibition of infection by 200 μg heparin or after treatment of SK6 cells with 12.5 mIU of heparinase I per ml. Percentages are the mean of at least two independent observations.

[d] Size of plaques observed after 2 days of growth in a plaque assay using SK6 cells. Cells were not treated with heparinase I, and no heparin was added.

[e] ND, not determined.

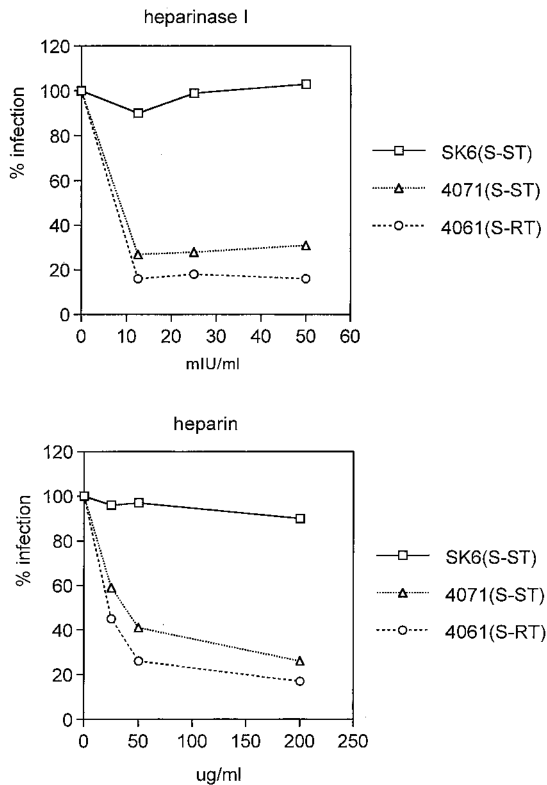
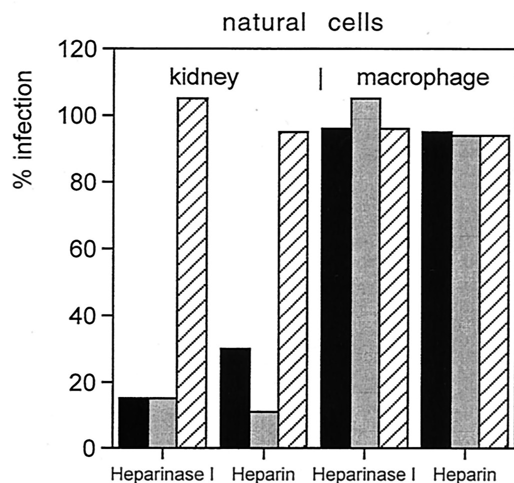


FIG. 2. Dose-dependent reduction of infection of SK6 cells with reisolated S-ST (blood from pig 4071) and S-RT (blood from pig 4061) viruses and with S-ST virus produced in SK6 cells [SK6(S-ST)] after heparinase I treatment of cells or by addition of 200 μg of heparin per ml to the infection medium. Tissue culture wells (2 cm^2) were infected with approximately 1,000 PFU of 4071 (S-ST) or 4061 (S-RT) virus and with 250 PFU of SK6(S-ST) virus. Blood samples taken on day 14 p.i. were tested. The number of plaques in wells was measured in a plaque assay, and the percent infection compared to that in control wells was calculated as described in Materials and Methods. Symbols represent the mean of two independent observations.

a dose-dependent manner (Fig. 2) whereas infection with the CoBrB.E^{rns}(S-ST) virus produced in SK6 cells [hereafter referred to as SK6(S-ST) virus] was not seriously affected. To exclude the possibility that the method of sampling results in an increased affinity of virus for HS, SK6(S-ST) virus was added to uninfected EDTA-treated blood of a pig. After two freeze-thaw cycles, infection with virus in this sample was not seriously affected by 200 μg of heparin per ml and by heparinase I treatment. In addition, infection with virus present in a freshly applied, non-freeze-thawed EDTA-treated blood and serum sample, taken from pig 4071 (S-ST) on day 14 p.i., was inhibited by 70 and 75%, respectively, after treatment of SK6 cells with 12.5 mIU of heparinase I per ml. These results indicated that the S-ST virus produced in pigs is indeed able to infect SK6 cells by an HS-dependent mechanism. To further show that this different behavior of in vivo-generated S-ST virus compared to SK6(S-ST) virus is dependent on the environment in which the virus is produced, SK6 cells were infected with blood of pig 4071 (14 days p.i.) to produce a p1 virus stock. When grown for 2 days under methylcellulose, this virus stock produced only large plaques indicating that E^{rns}S-ST was the predominant genotype of this p1 virus stock. In contrast to in vivo-generated S-ST virus, infection with this virus stock was not seriously inhibited by 200 μg of heparin per ml and was not affected by treatment of SK6 cells with 12.5 mIU of heparinase I per ml (results not shown). This indicated that the ability of the S-ST virus to infect SK6 cells by an HS-dependent mechanism is dependent on the environment in which this virus is produced.

Infection of native pig cells with in vivo- and in vitro-generated virus. The ability of in vivo-and in vitro-generated CSFV to infect native pig cells by an HS-dependent mechanism was studied. Inhibition or reduction of infection of primary kidney cells and lung macrophages by 200 μg of heparin per ml or after treatment of these cells with 20 mIU of heparinase I per ml was measured in plaque assays (Fig. 3). Similarly, as observed for infection of SK6 cells, viruses in the blood of pigs 4071 (S-ST) and 4061 (S-RT) were able to infect primary kidney cells by an HS-dependent mechanism whereas SK6(S-ST) virus infected these cells by an HS-independent mechanism. In contrast, infection of lung macrophages with SK6(S-ST) virus and with the in vivo-generated S-ST and S-RT viruses was not seriously inhibited by heparin or affected by heparinase I treatment of cells. It is likely that irrespective of the E^{rns} genotype (S-ST or S-RT), infection of lung macrophages with CSFV proceeds by an HS-independent mechanism (see Discussion). Titer determination of the SK6(S-ST) virus stock showed that the titer measured on macrophages was 100-fold higher than the titer measured on primary swine kidney cells (table in Fig. 3). In contrast, when blood of pig 4071 (S-ST) was subjected to titer determination, equal titers were measured on primary kidney cells and macrophages.

Surface properties of in vivo- and in vitro-generated virus. Earlier experiments (19) showed that infection of SK6 cells with clone 1.1.1 of CSFV strain Brescia (E^{rns} genotype R-RI [34]) was inhibited in a dose-dependent manner by ConA. ConA is a lectin that specifically binds with high affinity to terminal mannose (and with less affinity to glucosamine) residues of N-linked glycosyl groups. ConA binding was used as tool to scan the surface of in vivo-generated (blood of pig 4061 and 4071) and in vitro-generated E^{rns} virus variants in order to demonstrate possible differences in surface structure between these viruses. Appropriate dilutions of these viruses were preincubated with different concentrations of ConA for 30 min at 37°C. Preincubation mixtures were used to infect 2-cm^2 tissue culture wells with SK6 cells for 30 min at 37°C. Virus was removed from the cells, which were then treated with 100 mM MMP to remove residual ConA. ConA attached to the surface of SK6 cells inhibits cell-to-cell spread of CSFV (result not shown). To obtain plaques with a similar size, at all tested concentrations of ConA, this treatment with MMP was needed before the cells were overlaid. After 24 h of growth under methylcellulose, the cells were stained and plaques were counted (Fig. 4). For viruses produced in SK6 cells, virus secreted in the medium (cell-free virus) and virus associated with cells were tested separately. For all virus variants, no significantly different inhibition or stimulation of infection was observed between cell-free and cell-associated preparations. Therefore, results are shown for cell-free virus preparations. ConA inhibited infection with C1.1.1 [Fig. 4, SK6(R-RI)] in a dose-dependent manner. A concentration of 3 μg of ConA per



virus	titer on cell type (log 10 PFU./ml)		
	macrophage	kidney	SK6
4071(S-ST)	6,5	6.5	7.4
4061(S-RT)	6.0	7.0	7.7
SK6(S-ST)	6.4	4.3	4.7

FIG. 3. Reduction of infection of primary swine kidney cells and lung macrophages with reisolated S-ST (blood from pig 4071) and S-RT (blood from pig 4061) viruses and with S-ST virus produced in SK6 cells [SK6(S-ST)] after treatment of cells with 20 mIU of heparinase I per ml or by addition of 200 μg of heparin per ml to the infection medium. Tissue culture wells (2 cm^2) containing primary swine kidney cells were infected with approximately 300 PFU of 4071 (S-ST), 1,000 PFU of 4061 (S-RT), or 250 PFU of SK6(S-ST) virus. Wells with macrophages were infected with approximately 400 PFU of 4071 (S-ST), 1,000 PFU of 4061 (S-RT), or 400 PFU of SK6(S-ST) virus. Blood samples taken on day 14 p.i. were tested. The number of plaques in wells was measured in a macrophage plaque assay or in standard plaque assay (for primary kidney cells), and the percentage infection compared to control wells was calculated as described in Materials and Methods Bars represent the mean of three independent observations. Virus titers (log_{10} PFU per milliliter) in the blood of pigs 4071 and 4061, and SK6(S-ST) virus were measured in a macrophage plaque assay and in a standard plaque assay using cultured (SK6) or primary swine kidney (kidney) cells (table).

ml slightly stimulated infection with in vivo-generated S-RT virus [Fig. 4, 4061(S-RT)]. However, 50 μg of ConA per ml inhibited infection with this virus to the same extent as it did for CoBrB.E^{rns}(S-RT) virus produced in SK6 cells [SK6(S-RT)]. Surprisingly, infection with both S-ST viruses was stimulated. However, infection with SK6(S-ST) virus was stimulated 12-fold by 12 μg of ConA per ml whereas this concentration of ConA stimulated infection with in vivo-generated S-ST virus [4071(S-ST)] no more than 2- to 3-fold. Addition of MMP (40 mM) to preincubation mixtures containing 12 μg of ConA per ml abrogated the stimulation of SK6(S-ST) virus and the inhibition of C.1.1.1 virus [SK6(R-RI)] completely (Fig. 4). This indicated that binding of ConA to N-linked glycans exposed on the surface of virions is responsible for the observed stimulation or inhibition of infection. The significantly higher stimulation of infection observed for the SK6(S-ST) virus than for its in vivo-generated counterpart indicated that these S-ST genotypes have different surface properties.

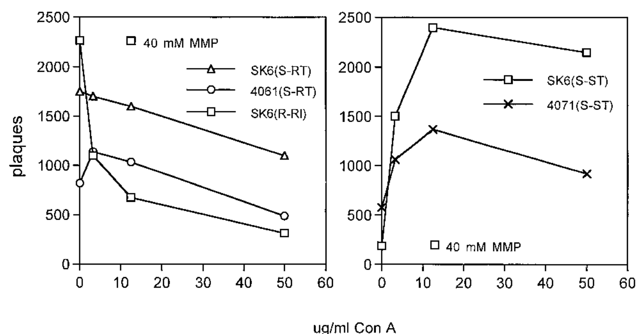


FIG. 4. Inhibition or stimulation of infection by ConA. Viruses were preincubated with different concentrations of ConA for 30 min at 37°C. Preincubation mixtures were used to infect 2-cm^2 tissue culture wells with SK6 cells for 30 min at 37°C. After removal of virus and washing of cells, the cells were incubated with 100 mM MMP for 30 min at 37°C. Subsequently, they were washed and supplied with overlay medium and stained after 24 h of growth at 37°C. Symbols represent the mean number of plaques calculated from two independent observations.

Characterization of E^{rns} proteins. Using the baculovirus system, an S-RT and an S-ST version of E^{rns} (strain Brescia) were expressed in insect cells and purified from these cells using immunoaffinity chromatography (15). Characterization of these purified proteins (17) showed that both recombinant proteins were correctly expressed. In reducing and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, both proteins migrated similarly fast. Moreover, both proteins were expressed as homodimers. Also, they had an RNase activity comparable to that of native E^{rns} (results not shown). To investigate whether these proteins are able to bind to a heparin-HS-type polysaccharide chain, purified S-ST and S-RT proteins were applied to heparin-Sepharose columns (heparin isolated from porcine intestinal mucosa coupled to Sepharose-4B) at 0 mM NaCl. By increasing the concentration of NaCl stepwise, proteins were eluted. Similarly to the situation reported for E^{rns} of CSFV strain C (S-RT) (18), the S-RT protein eluted as a broad peak at a relatively high concentration of NaCl (ca. 700 mM) (Fig. 5). The S-ST protein also eluted as a broad peak but at a lower concentration of NaCl (ca. 400mM) than the S-RT protein did. (Fig. 5). This indicated that E^{rns} S-ST was also able to bind tightly to heparin-HS

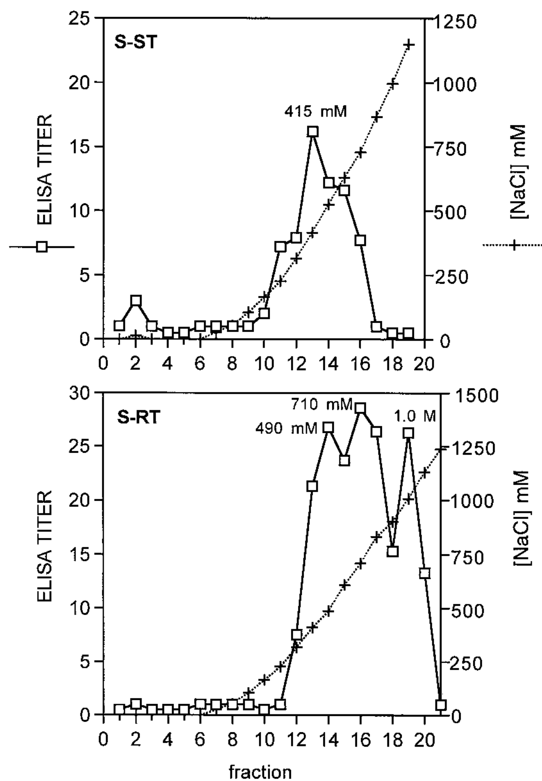


FIG. 5. Heparin-Sepharose chromatography of E^{rns}. Purified E^{rns} S-ST or E^{rns} S-RT was loaded onto the column at a concentration of 0 mM NaCl. Proteins were eluted by increasing the NaCl concentration stepwise. The fractions were assayed for E^{rns} in an ELISA, and the NaCl concentration of these fractions was determined by measuring the osmolarity.

type polysaccharide chains. Moreover, when SK6 cells were incubated with these recombinant proteins, binding to the cell surface of SK6 cells could be detected for E^{rns} S-RT and S-ST (16). The fact that practically no cell surface staining was observed for either protein in the presence of 200 μg of heparin per ml indicated that E^{rns} S-RT and S-ST interacted specifically with membrane-associated HS (results not shown).

DISCUSSION

In cell culture selected virus variants of FMDV (39) and SV (27), carrying mutations that confer HS attachment, are attenuated in animals. We therefore expected that the swine kidney cell-selected HS-dependent CSFV variant, carrying an Arg in the C terminus of E^{rns}, would be less virulent than its HS-independent counterpart with a Ser at this position. However, the results presented here indicated that the adaptive Ser-to-Arg mutation did not reduce virulence. Similarly, as observed for pigs infected with native virus and CoBrB.E^{rns}(S-ST) recombinant virus, the three pigs infected with the CoBrB.E^{rns} (S-RT) recombinant virus became seriously ill and developed characteristic symptoms of acute CSFV. Moreover, no E2 antibody response was induced, which is correlated with clearance of virus, and all three pigs stayed viremic until death or until the end of the trial.

The dose administered to pigs was based on titers determined using SK6 cells. For CoBrB.E^{rns}(S-ST), a 100-fold-higher titer was measured when macrophages instead of SK6 cells were used for titer determination (Fig. 3). Therefore, it is possible that S-ST-infected pigs received much more virus than did pigs infected with the CoBrB.E^{rns}(S-RT) virus. Consequently, this could account for the observed slightly faster onset of disease observed in S-ST pigs. However, more pigs per group are needed to correlate this properly.

Passage of HS-dependent FMDV variants through cattle showed that these variants rapidly mutated to more virulent viruses which have no or reduced affinity for HS or heparin (39). In blood samples from one of the pigs infected with CoBrB.E^{rns}(S-RT), a low level of a large-plaque phenotype could be detected. Efficient replication of CoBrB.E^{rns}(S-RT) in pigs probably prohibited further enrichment of this large-plaque phenotype in this pig and detection in the two other pigs. Nevertheless, the existence of these plaques indicated that in this pig, CoBrB.E^{rns}(S-RT) reverted to a variant with a lower affinity for HS, most probably by selection in specific host cells in which CoBrB.E^{rns}(S-RT) replicates poorly. Detection of a Ser^{476} codon instead of an Arg^{476} codon in viral RNA isolated from such a large-plaque further supported this argument.

Analysis of virus isolated from pigs infected with CoBrB.E^{rns} (S-ST) virus indicated that replication in vivo induced no mutations in the genes encoding the three envelope proteins. Thus, virus samples from these pigs provided us with a homogeneous population of in vivo-generated S-ST virus that is representative for its parental field isolate (18, 49). The results presented here indicated that in vivo-generated CSFV, carrying an E^{rns} S-ST protein on its envelope, is able to use membrane-associated HS as attachment receptor and that HS-dependent infection of more natural (primary) cells could take place. However, infection of lung macrophages with in vivo-generated S-ST virus, and even with the HS-dependent S-RT virus variant, was not affected by heparin and heparinase I treatment. Macrophages do express HS chains on their cell surface (22). Therefore, this might indicate that attachment of CSFV to HS chains on the surface of macrophages is bypassed by an interaction with another surface molecule. The fact that HS-independent S-ST virus produced in SK6 cells infected macrophages more efficiently than it infected primary and cultured swine kidney cells (Fig. 3) suggested that this molecule is not abundantly expressed on the surface of kidney cells. This HS-independent attachment may represent a crucial event for entry of CSFV into specific cells of the host and might be mediated by interaction of specific domains exposed on the surface of virions. Surface domains are probably distinct from the E^{rns} domains that confer HS binding.

One round of amplification of animal-derived S-ST virus in SK6 cells changed this HS-dependent virus population to a population that reacted independently of HS. This is consistent with our previous hypothesis that the surface structure of in vivo-generated S-ST virus particles is distinct from that of S-ST particles produced in SK6 cells (18). The lower level of ConA stimulation of infection for in vivo-generated compared to in vitro-generated S-ST virus supported this argument. However, the mechanism of ConA stimulation is not completely clear. ConA specifically binds with high affinity to terminal mannose residues of N-linked glycosyl groups (and with a lower affinity

to terminal glucosamine residues). No data are available on the structure of the N-linked glycosyl groups that are predominantly exposed on the surface of CSFV particles. However, ConA binding to CSFV variants indicated that N-linked glycans with terminal mannose residues are present on the surface of virions. Processing of N-linked glycans, including addition of nonsugar residues, linked to these chains (e.g., sulfate and phosphate groups [23, 29]) is dependent on the routing of proteins through the exocytotic pathway (29, 36) and can be dependent on cell-specific factors (20, 23, 29). Due to replication in various cell types, the structure of N-linked glycans on the surface of in vivo-generated S-ST virus particles may be more variable and distinct from that of S-ST virus particles produced in a homogeneous environment like SK6 cell cultures. For instance, the number of negatively charged residues linked to the glycan chains and the positions where these groups are linked can affect the distribution of charges on the surface of virus particles. An overall (or locally) higher net negative charge could enhance the repulsion from the sulfate-rich HS clusters (9, 26, 44), which are most probably essential for the attachment of HS-dependent CSFV to the surface of SK6 cells (18). Binding of ConA to glycans on the surface of S-ST virus could mask these negative charges and increase the binding to HS and, consequently, infection. S-ST particles produced in SK6 cells may expose more of these negatively charged residues than in vivo-generated S-ST virus particles do, resulting in the observed higher level of stimulation of infection. In line with this hypothesis, ConA binding to the R-RI variant could mask positively charged domains involved in attraction to HS, resulting in inhibition of infection. Interestingly, ConA inhibition or stimulation of virus variants produced in cell culture exactly correlated with the charge of their E^{rns} amino acid backbone (from positive to negative, R-RI, S-RT, and S-ST [18]). Moreover, it also correlated with the ratio of secreted (cell-free) to cell-associated virus particles produced in SK6 cell cultures (R-RI, 0.05; S-RT, 1.5; S-ST, 3 [results not shown]).

In a previous study, reduced particle release from SK6 cells was inversely correlated with the virulence of CSFV strains (32). Most of the S-RT virus particles do not stay cell associated. These particles are probably not sequestered to sites in pigs that are unfavorable for replication (27, 39), and they are able to spread efficiently in pigs. This may explain the replication competence of this virus in vivo. However, this could be different for the more cell-associated R-RI variant. Therefore, the effect of the N-terminal Ser^{276}-to-Arg and C-terminal Thr^{477}-to-Ile mutations in E^{rns} on HS binding and replication in vivo is currently under investigation. Interestingly, the N-terminal mutation abrogates a potential N-linked glycosylation site (NXS) (18, 29).

Besides glycosylation, other (nongenetic) structural differences may account for the higher binding affinity of in vivo-generated S-ST virus for swine kidney cells compared to in vitro-generated S-ST virus. Due to exposure to rough environments (e.g., host enzymes) or interactions with host factors, the surface of in vivo-generated virus particles may be remodeled. Association of E^{rns} with the envelope is tenuous (11), and virus particles may lose E^{rns} molecules from their surface. These events could modify the surface structure and/or the distribution of charges on the surface of virions (see above). Spiking of blood with SK6(S-ST) virus and direct testing of fresh blood and serum revealed that two freeze-thaw cycles did not resulted in HS binding of SK6(S-ST) virus to the surface of SK6 cells. However, we cannot completely rule out the possibility that some of the E^{rns} molecules are lost from the envelope during sampling, handling, and testing of in vivo-generated virus. Recently, immunoelectron microscopic studies revealed that E2 molecules on the surface of virus particles were detected only after virus particles were released from the cell surface (48). Perhaps interaction with HS withholds weakly bound E^{rns} from virions. This may trigger virus release from the cell surface and could result in the exposure of E2 domains. Because interaction of E2 with the cell surface is involved in virus entry (16, 55), this process may be essential for the production of an infection-competent CSFV particle. However, modification of the envelope proteins by specific host cell factors late in morphogenesis may also be responsible for virus release and exposure of E2 domains. Such activation mechanisms have been reported for other viruses (26). Interestingly, for SV it was shown that the basic protease cleavage signal in the N terminus of envelope protein E2 was involved in direct binding to HS (28). Cleavage of this site by furin protease releases mature E2 from its PE2 precursor. In mosquito cells, in which cleavage of this HS-binding motif was more efficient than in BHK cells, virus with a reduced content of PE2 and, consequently, with a reduced affinity for cell surface HS was produced (28). This demonstrated that the surface properties of virus particles can be dependent on cell-specific processing events.

When kidney and tonsil samples from S-RT pigs were subjected to titer determination, a dramatically lower virus titer was established when macrophages instead of SK6 cells were used for titer determination (Table 2). This difference was less apparent when blood samples from S-RT pigs were used. In contrast, the ratio of SK6 to macrophage titers for S-ST virus samples was almost equal for blood and tissue samples. This suggests that in vivo-generated S-RT particles did bind less efficient to the surface of macrophages than did in vivo-generated S-ST particles. In addition, it suggested that the surface of S-RT virus particles produced in kidney and tonsil cells might differ from that of virus particles present in the blood of S-RT pigs, which are most probably produced by various types of cells. Nevertheless, the virus load in tonsil and kidney preparations was significantly higher for S-RT virus than for S-ST virus. This higher virus load indicated that acquisition of an extra Arg in the C terminus of E^{rns}, induced by passage in epithelium-like cells (SK6), enhanced the infection of similar types of cells in vivo. A close resemblance of SK6 cells to cells present in kidneys and tonsils may also be an explanation for the observed lower S-ST viral load in these organs, in spite of the ability of this virus to infect these types of cells efficiently through HS binding. Primary replication of S-ST virus in native cells, which are closely related to SK6 cells, may also produce a more homogeneous, fully processed population of viruses, which could be unable to infect nearby cells efficiently due to their failure to interact with HS. Additionally, most of these S-ST virus particles do not stay cell associated and may even be repelled from the surface of adjacent cells. This could result in an exodus of S-ST viruses from these organs and, consequently, in a relatively lower viral load. The results presented

here indicate that the efficiency of virus replication in specific tissues can be manipulated by adaptation of virus to closely related cell lines. Insertion of an HS-binding motif in the fiber protein of adenovirus also showed that the efficiency of infection of specific cells could be improved (53).

Detailed studies of the processing and surface structure of in vivo-produced virus are needed to further show that CSFV utilizes HS binding to infect cells in vivo and/or to associate with cells present in the body fluids of their host. Binding of virus to HS on the surface of these cells may be an efficient mechanism for CSFV to spread in its host. In addition, studies of the interactions of virus-bound and free (secreted) E^{rns} with HS and with an as yet unidentified additional receptor(s) are of great interest. Interaction of E^{rns} with its so-called low-affinity receptor, HS, may facilitate interaction with a more specific receptor on the surface of lymphocytes (41). Activation of such a receptor may initiate immune suppression (4, 31, 45).

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