

Vesicular Stomatitis Virus Glycoprotein Is a Determinant of Pathogenesis in Swine, a Natural Host

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There are two major serotypes of vesicular stomatitis virus (VSV), Indiana (VSIV) and New Jersey (VSNJV). We recovered recombinant VSIVs from engineered cDNAs that contained either (i) one copy of the VSIV G gene (VSIV-G^I); (ii) two copies of the G gene, one from each serotype (VSIV-G^{NJ}G^I); or (iii) a single copy of the G^{NJ} gene instead of the G^I gene (VSIV-G^{NJ}). The recombinant viruses expressed the appropriate glycoproteins, incorporated them into virions, and were neutralized by antibodies specific for VSIV (VSIV-G^I), VSNJV (VSIV-G^{NJ}), or both (VSIV-G^{NJ}G^I), according to the glycoprotein(s) they expressed. All recombinant viruses grew to similar titers in cell culture. In mice, VSIV-G^{NJ} and VSIV-G^{NJ}G^I were attenuated. However, in swine, a natural host for VSV, the G^{NJ} glycoprotein-containing viruses caused more severe lesions and replicated to higher titers than the parental virus, VSIV-G^I. These observations implicate the glycoprotein as a determinant of VSV virulence in a natural host and emphasize the differences in VSV pathogenesis between mice and swine.

Vesicular stomatitis viruses (VSV) are members of the genus *Vesiculovirus* of the family *Rhabdoviridae*. They belong to the order *Mononegavirales*, which includes all the nonsegmented negative-strand RNA viruses. The 11-kb VSV genomic RNA is transcribed into five capped and polyadenylated mRNAs by the viral RNA-dependent RNA polymerase. The mRNAs encode five structural proteins: the nucleocapsid protein, N; the phosphoprotein, P, which is a cofactor of the RNA-dependent RNA polymerase, L; the matrix protein, M; and the attachment glycoprotein, G. The genes are arranged in the order 3'-N-P-M-G-L-5', and gene expression is obligatorily sequential from a single 3' promoter (1, 2). Due to transcriptional attenuation at each gene junction, the amount of mRNA transcribed is a function of gene position relative to the promoter. Promoter-proximal genes are transcribed at higher levels than more distal genes (23, 54).

VSV produces an acute disease in cattle, horses, and swine. The disease is characterized by vesiculation and ulceration of the tongue, oral tissues, feet, and teats, and the lesions are similar to those seen in foot-and-mouth disease, one of the most devastating animal diseases (44). VSV causes important economic losses due not only to decreased milk and meat production but also to quarantines, trade barriers, and livestock market closures (35).

Two major serotypes of VSV, New Jersey (VSNJV) and Indiana (VSIV), have been described based on neutralizing antibodies to the surface glycoprotein, G (7, 25). There is only 50% identity at the amino acid level between the G proteins of

VSIV and VSNJV. The VSIV G protein (G^I) has 511 amino acids, is glycosylated at positions 178 and 335, and contains covalently linked fatty acid in the cytoplasmic domain (9, 46). The VSNJV glycoprotein (G^{NJ}) contains 517 amino acids, and the glycosylation sites are identical to those of G^I, but it is not acylated (17). The VSIV and VSNJV glycoproteins also differ in their antigenic structures (24). In general, neutralizing monoclonal antibodies do not cross-react between the G proteins of the two serotypes. One epitope on the G^I protein was defined by a monoclonal antibody that could bind to the G proteins of both serotypes, but it could neutralize the infectivity of only VSIV (29). Many nonneutralizing antibodies, both cross-reactive and serotype specific, have also been described (4, 30). The VSV G^I protein also contains T helper epitopes (6) and induces a cross-reactive cytotoxic-T-lymphocyte response (19). In addition to the primary sequence and antigenic structure, the G^I and G^{NJ} glycoproteins differ in maturation. The G^{NJ} glycoprotein folds faster intracellularly and with less dependence on glycosylation than does G^I (33, 34).

Of the two major serotypes, VSNJV strains are economically more important than VSIV strains, since they are responsible for most of the epizootics and their pathogenicities have been shown to be greater than those of VSIV strains (5, 43, 51). However, isolates of these serotypes have been studied only superficially for differences in biological properties, and the molecular determinants of VSV pathogenesis in natural hosts remain undefined. Reverse genetic techniques, along with a domestic pig model for VSV infection (14), allowed us to test the hypothesis that the G glycoprotein was involved in the differential pathogenesis of VSIV and VSNJV in swine, one of the natural hosts for VSV. VSIV cDNA clones previously constructed in our laboratory (56, 58) were used to generate engineered VSIVs that differed only in their G genes. The G glycoprotein was chosen because important differences in structure, maturation, and antigenicity between serotypes have

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been reported (see above). Recovered viruses were characterized as to protein expression, assembly, and replication in cell culture. We inoculated these recombinant viruses into pigs and analyzed their pathogenicities as measured by the severity of the induced lesions and by virus replication. The results were compared to those obtained after intranasal inoculation of mice, a well-established laboratory model for VSV pathogenesis (21, 36, 38, 40, 48, 55). Our observations demonstrated the involvement of the G glycoprotein in the pathogenesis of VSV in swine and highlighted the differences between laboratory mice and natural hosts following VSV infection.

MATERIALS AND METHODS

Viruses and cells. The San Juan isolate of the Indiana serotype of VSV provided the template for all of the cDNA clones of the VSV genome except the G protein gene, which was derived from either the Orsay (VSIV) (58) or 95COB (VSNJV) isolate. Isolate 95COB was obtained from a bovine during an outbreak of VSNJV in Colorado in 1995 (45). Baby hamster kidney (BHK-21) cells were used to recover viruses from cDNAs, for single-step growth experiments, and for radioisotopic labeling of viral proteins. Vero-76 cells were used for plaque assays.

Plasmid construction and recovery of infectious virus. Full-length cDNA clones of the VSIV genome containing an additional heterologous transcriptional unit cloned under *Xho*I restriction sites at each of the four internal gene junctions and flanked by the conserved gene start signal and 3' untranslated region and the gene end of the G^I gene were previously described (56). The plasmid containing the transcriptional unit (I) at the M-G junction (pMIG) was used to clone the G^{NJ} gene from the VSNJV 95COB strain to generate the VSIV-G^{NJ}G^I recombinant (Fig. 1A). The coding region of the G^{NJ} gene was amplified by reverse transcription (RT)-PCR from purified viral RNA using two oligodeoxynucleotides containing *Xho*I restriction sites (5' TGACTCGAGATCAATATGTTGTC3' and 3' GGGTGAAGGCAATCGAGCTCAGT5'; *Xho*I recognition sites are underlined, and start and stop codons are in boldface). This RT-PCR product was cloned into the PCR-Blunt II-Topo plasmid (Invitrogen) and used to replace the transcriptional unit in the pMIG plasmid by digestion with *Xho*I. The same RT-PCR product was cloned into a VSIV plasmid lacking the G gene (pΔG) to generate the VSIV-G^{NJ} recombinant (Fig. 1A). The presence and position of the inserted gene were confirmed by restriction enzyme analysis and sequencing of the insertion sites in the plasmids. To recover infectious virus, BHK-21 cells were infected with the vaccinia virus recombinant that expresses T7 RNA polymerase (vTF7-3) and transfected with the full-length cDNA clones and three support plasmids that expressed the N, P, and L proteins required for RNA encapsidation and replication (3, 58). Infectious viruses were recovered from the supernatant medium, amplified by passage on BHK-21 cells at low multiplicity in the presence of 1-β-D-arabinofuranosylcytosine to inhibit replication of vTF7-3, and filtered through 0.2-μm-pore-size filters. Virus stocks were tested for the presence of vTF7-3 by neutralization with VSIV- or VSNJV-specific antibodies. No vTF7-3 plaques were detected in up to 3 × 10⁶ PFU of recombinant VSIVs.

Analysis of virion proteins. To assess proteins in mature virions, BHK-21 cells were infected at a multiplicity of infection of 5 PFU/cell. At 2 h postadsorption, the cells were washed and incubated in methionine-free medium for 30 min. The cells were labeled with [³⁵S]methionine (50 μCi/ml) overnight. The supernatant fluid was collected, cell debris, was removed by low-speed centrifugation, and the virus was collected by centrifugation through a 10% sucrose cushion. The viral pellet was resuspended in 0.01 M Tris, pH 7.5, and the virion proteins were solubilized in gel loading buffer (27) and separated on a 10% polyacrylamide gel. Viral proteins were quantitated by densitometric analysis of autoradiographs of gels, and molar ratios were calculated after adjustment for the methionine content. Comparisons between lanes were done after normalization against the nucleoprotein.

Neutralization of recombinant viruses with antibodies specific for Indiana or New Jersey serotype. Serial threefold dilutions of antibodies specific for VSIV (ATCC VR-1238AF) or VSNJV (ATCC VR-1239AF) in Dulbecco's modified Eagle medium plus 2% fetal calf serum (DMEM2) were mixed with an equal volume of the same medium containing 200 PFU of the corresponding virus. After 30 min of incubation at 37°C, the virus-antibody mixture was added to confluent Vero-76 cells growing in six-well dishes. Virus was adsorbed for 1 h, the inoculum was removed, and the cells were washed twice, covered with a nutrient agarose overlay, and incubated for 16 to 18 h at 37°C. The cell monolayers were

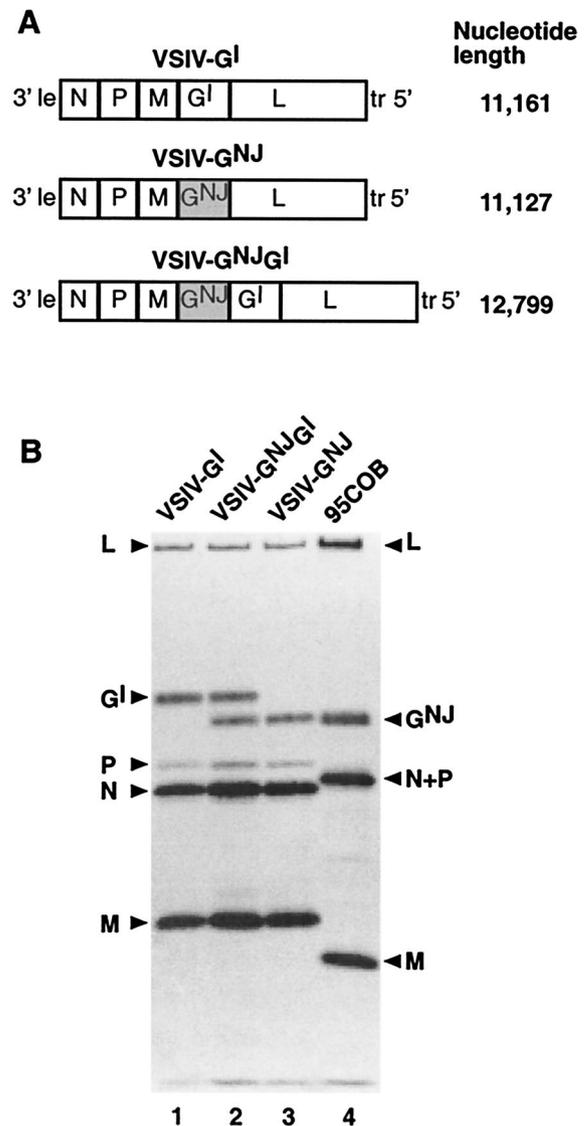


FIG. 1. (A) Schematic representation of recombinant VSV genomes. The negative-strand recombinant VSIV genomic RNAs indicating the gene order and expressed proteins are shown. VSIV leader (le), trailer (tr) and viral (N, P, M, G^I, and L) genes are represented. G^{NJ} refers to the glycoprotein gene from the VSNJV field isolate 95COB (shaded boxes). The lengths of the genomes are shown on the right. (B) Viral proteins incorporated in virus particles. Virions were labeled with [³⁵S]methionine and purified by centrifugation through 10% sucrose, and the virion proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The infecting viruses are indicated above the lanes, and synthesized proteins are indicated on the left for the recombinant VSIV viruses and on the right for 95COB.

fixed with formaldehyde and stained with crystal violet, and the virus plaques were counted.

Single-cycle virus replication. Single-step growth analysis was conducted in BHK-21 cells infected at a multiplicity of infection of 3 PFU/cell. After 1 h of adsorption, the inoculum was removed and the monolayers were washed twice. Fresh medium was added, and the cells were incubated at 37°C. Supernatant fluids were harvested at appropriate intervals, and viral yields were determined by plaque assay on Vero-76 cells.

Lethality in mice. Three-week old male Swiss Webster mice were purchased from Taconic Farms, Germantown, N.Y., and housed under biosafety level 3 (BL-3) containment conditions. Groups of 10 mice were lightly anesthetized with ketamine-xylazine and inoculated intranasally with 10 μl of 10-fold dilutions of

the individual viruses in DMEM2. Control animals were given an equal volume of DMEM2. Following inoculation, the virus preparations were all within three times the original titer as confirmed by plaque assay. The animals were observed every day, and the 50% lethal dose (LD_{50}) for each virus was calculated by the method of Reed and Muench (39).

Swine inoculation and sample collection. Twenty-four Yorkshire pigs (8 to 10 weeks old and weighing 25 to 30 kg) were obtained from a local breeder and housed under BL-3 isolation conditions at the Plum Island Animal Disease Center. All animals were negative for VSIV and VSNJV, as indicated by serum neutralizing assay. The pigs were divided into two groups of four (for inoculation with VSIV-G^I or 95COB) and two groups of eight (for inoculation with VSIV-G^{NJ}G^I or VSIV-G^{NJ}), and each group was housed in a separate room under BL-3 isolation conditions. The epidermis of the snout in pigs that had been sedated with xylazine-ketamine-telazolol was pricked 20 times using a dual-tip skin test applicator (Duotip-Test; Lincoln Diagnostics, Decatur, Ill.), and 3×10^6 PFU of virus was placed on the scarified area in 100 μ l of DMEM2. The area under the inoculum was then resensitized by repeating the scarification procedure, and the animals were restrained in a stationary position until the inoculum was adsorbed. Virus preparations were all within three times the original titer following the inoculation procedure. The temperature and appearance of the animals were monitored daily, and samples were collected by individuals wearing protective clothing and portable HEPA respirators, which were changed for entry into each isolation room. Esophageal-pharyngeal fluid (EPF) was obtained before and at various times following virus inoculation by using a modified pharyngeal or probang scraper. Nasal swabs and serum samples were taken before and at various times following inoculation. Swabs of any lesions that appeared were also taken and tested by virus isolation. Swabs and EPF samples were collected in antibiotic-containing basal medium Eagle plus 1% fetal calf serum.

Assessment of clinical disease in swine. The extents of disease resulting from inoculation with the different viruses were evaluated by assessing lesion formation using a clinical scoring system based on the sizes and locations of the resulting lesions. Scores were assigned as follows: 0 indicated no visible lesions, 1 was given for a lesion that occurred at the site of inoculation and was <2 cm in diameter, 2 indicated a lesion that was >2 cm in diameter or multiple lesions at the site of inoculation, 3 indicated a lesion of <2 cm at a noninoculation site, and 4 indicated a lesion of >2 cm or multiple lesions at noninoculation sites.

Titration of virus isolated from pigs. Nasal swabs, EPF, and lesion swabs were analyzed for the presence and titer of virus by inoculation onto BHK-21 cell monolayers in 96-well plates after the samples had been clarified by centrifugation. The supernatants were serially diluted and added to the cells in quadruplicate wells, the plates were incubated at 35.5°C for 3 days, and the 50% tissue culture infective dose was calculated.

RESULTS

Generation and recovery of recombinant viruses. We generated recombinant VSV based on the Indiana serotype by engineering infectious cDNA clones in which the homologous G^I open reading frame (ORF) was replaced by the G^{NJ} ORF or in which the G^{NJ} ORF was added to yield a VSIV genome containing both the G^I and G^{NJ} ORFs, as described in Materials and Methods and shown schematically in Fig. 1A. These viruses, along with the wild-type Indiana recombinant (VSIV-G^I) (58) and the New Jersey field isolate 95COB (45), were used throughout this work. All experiments involving VSNJV 95COB were carried out under appropriate biological containment at the U.S. Department of Agriculture Plum Island Animal Disease Center.

The RNA genomes of viruses recovered from cDNA were characterized by sequence analysis after RT-PCR. The N, P, M, G^I, and G^{NJ} genes and the corresponding gene junctions were sequenced to confirm the insertion of the G^{NJ} gene from VSNJV 95COB and to determine whether changes in sequence occurred during the recovery process. The order of genes (Fig. 1A) and the sequence of the intergenic junctions were as in the original cDNAs, and the N, P, M, and G^I sequences were identical to those of the parental VSIV-G^I.

There were two nucleotide differences in the G^{NJ} gene in the recombinant viruses compared to that in 95COB that caused the amino acid changes K113M and P238S. These changes were present in the original G^{NJ} DNA inserted in the two clones. As shown below, these mutations did not alter the antigenicity of the protein and do not map within any reported domain important for structure or functionality, such as glycosylation sites or domains involved in folding, trimerization, intracellular transport, or fusion (13, 16, 17, 32, 50, 59, 60).

Virion proteins. The synthesis of viral proteins was examined in BHK-21 cells by metabolic labeling with [³⁵S]methionine. Recombinant viruses expressed the expected viral proteins: five in the case of VSIV-G^I and VSIV-G^{NJ} and six in the case of VSIV-G^{NJ}G^I, which expressed both G^{NJ} and G^I glycoproteins (data not shown). The protein content of virus particles produced during infection of BHK-21 cells was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the [³⁵S]methionine-labeled proteins from purified viruses. Each virus contained the appropriate proteins, and VSIV-G^{NJ}G^I incorporated both G^{NJ} and G^I glycoproteins into virions (Fig. 1B). The G^{NJ} protein electrophoretic mobility was slightly faster than that of the G^I protein, as described previously (29). VSIV-G^I and VSIV-G^{NJ}G^I virions accumulated similar total amounts of G protein in their envelopes. This amount was ~20% lower in VSIV-G^{NJ} when normalized to the amounts of N protein in the respective virions. The amount of the G^{NJ} protein incorporated in the virus particles was always slightly lower than the amount of G^I protein (Fig. 1B, lanes 1, 2, and 3). In VSIV-G^{NJ}G^I virions, the amounts of the G^{NJ} and G^I proteins accounted for 40 and 60% of total G protein, respectively. The VSNJV (95COB) field isolate incorporated a larger amount of G in its virions than any of the recombinant VSIVs (Fig. 1B, lanes 1 through 4). Interestingly, this virus replicated faster than the recombinant viruses in BHK-21 cells, and it was also the most pathogenic in mice and natural hosts (see below).

Neutralization of recombinant viruses by antibodies against Indiana or New Jersey serotype. To determine whether the recombinant viruses could be neutralized by antibodies specific to the VSIV or VSNJV serotype, according to the glycoprotein(s) they expressed, the viruses were incubated with neutralizing antibodies against VSIV or VSNJV before inoculation of Vero-76 cells (Fig. 2). As expected, VSIV-G^I was completely neutralized by the VSIV-specific antibodies but not by the VSNJV-specific antibodies. Similarly, VSIV-G^{NJ} was neutralized by antibodies against VSNJV but not by antibodies against VSIV (Fig. 2). VSIV-G^{NJ}G^I was neutralized by antibodies against either VSIV or VSNJV. However, differences were observed in the neutralization curves between VSIV- and VSNJV-specific antibodies. Antibodies against VSIV completely neutralized VSIV-G^{NJ}G^I at the lower dilutions, indicating that the G^{NJ} and G^I glycoproteins were expressed in the same virion. Antibodies against the VSNJV serotype neutralized VSIV-G^{NJ}G^I, but neutralization was not complete at the lowest dilution tested. This was consistent with the observation that the amount of G^I in these virions was higher than that of G^{NJ} (Fig. 2) and suggests that enough G^I homo-oligomers may form in some virions to allow infection in the presence of VSNJV-specific antibodies or that some virions may have only G^I.

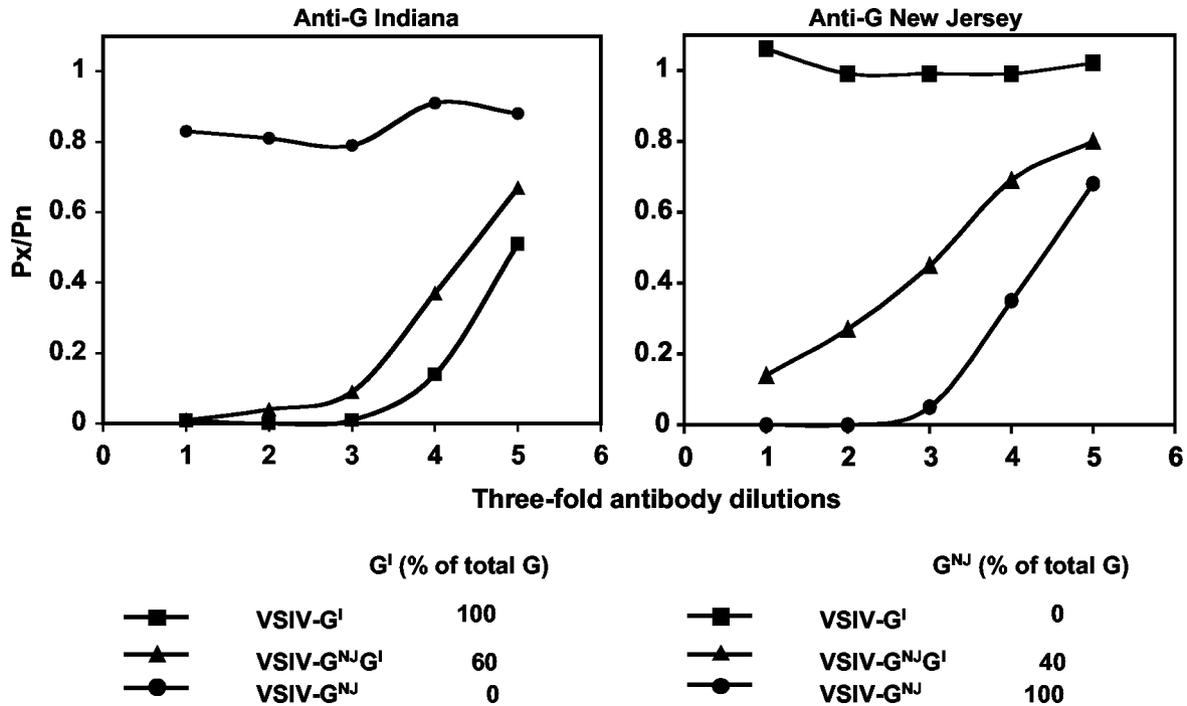


FIG. 2. Neutralization of recombinant and wild-type viruses by antibodies specific for Indiana or New Jersey serotype. Two hundred PFU of virus were incubated for 30 min at 37°C with serial threefold dilutions (starting at 1:40) of antibody prior to inoculation of Vero-76 cells. After 16 to 18 h of incubation at 37°C, individual plaques were counted. Px and Pn represent the numbers of plaques in the presence and absence of antibody, respectively. The percentages of G^I (VSIV) and G^{NJ} (VSNJV) in the total G protein present in the different virions, as determined in Fig. 1B, are indicated at the bottom.

Virus replication in cell culture. Replication of the recombinant viruses was examined under single-step growth conditions in BHK-21 cells at 37°C. Supernatant fluids were harvested at various times, and the virus yields were measured by plaque assay on Vero-76 cells (Fig. 3). Growth curves from VSIV-G^{NJ}G^I and VSIV-G^{NJ} were indistinguishable from that of VSIV-G^I (Fig. 3), showing that the ability of these viruses to replicate in cell culture was not impaired by the insertion of an additional gene or by the replacement of the G^I gene by the G^{NJ} gene. However, replication of recombinant viruses was delayed compared to that of the wild-type VSNJV (95COB) (Fig. 3). This difference was most apparent 90 min after the adsorption period, when the amount of 95COB virus in the supernatant was 100-fold greater than that of VSIV-G^{NJ}, as shown by a second experiment in which samples were taken every 30 min (Fig. 3, inset). After that, both viruses grew with similar kinetics and reached equivalent titers in the supernatant, indicating that the observed differences were most likely due to an early step in the infection cycle.

Lethality in mice. Young mice are susceptible to fatal encephalitis after intranasal inoculation of wild-type VSV and provide a well-studied model for comparing the relative lethalities of VSV and its mutants (36, 48, 55). Inoculation of mice allowed us to compare the pathogenicities of the wild-type and variant viruses in this animal model with those in swine, a natural host for VSV (14) (see below). We inoculated groups of 10 Swiss-Webster mice intranasally with serial 10-fold dilutions ranging from 1 to 10⁶ PFU per mouse. The mice were observed daily for clinical symptoms and death. The LD₅₀s were

11, 123, and >10⁶ PFU/mouse for VSIV-G^I, VSIV-G^{NJ}G^I, and VSIV-G^{NJ}, respectively. The morbidity and mortality of mice inoculated with 1,000 PFU of viruses are shown in Fig. 4. The time to onset of sickness and death increased progressively in

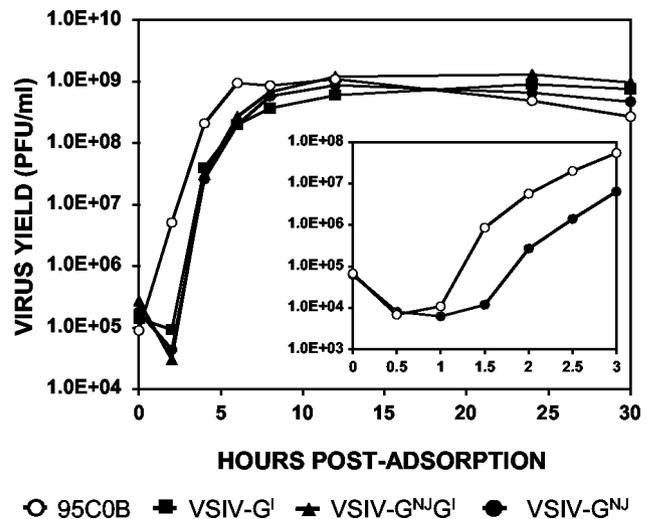


FIG. 3. Single-step growth curves of wild-type and recombinant viruses in BHK-21 cells at 37°C. The cells were infected at a multiplicity of infection of 3, and samples of the supernatant medium were harvested at the indicated time points. The samples were titrated in duplicate by plaque assay on Vero-76 cells. Single-step growth curves comparing VSIV-G^{NJ} and 95COB viruses at early times postinfection are shown in the inset.

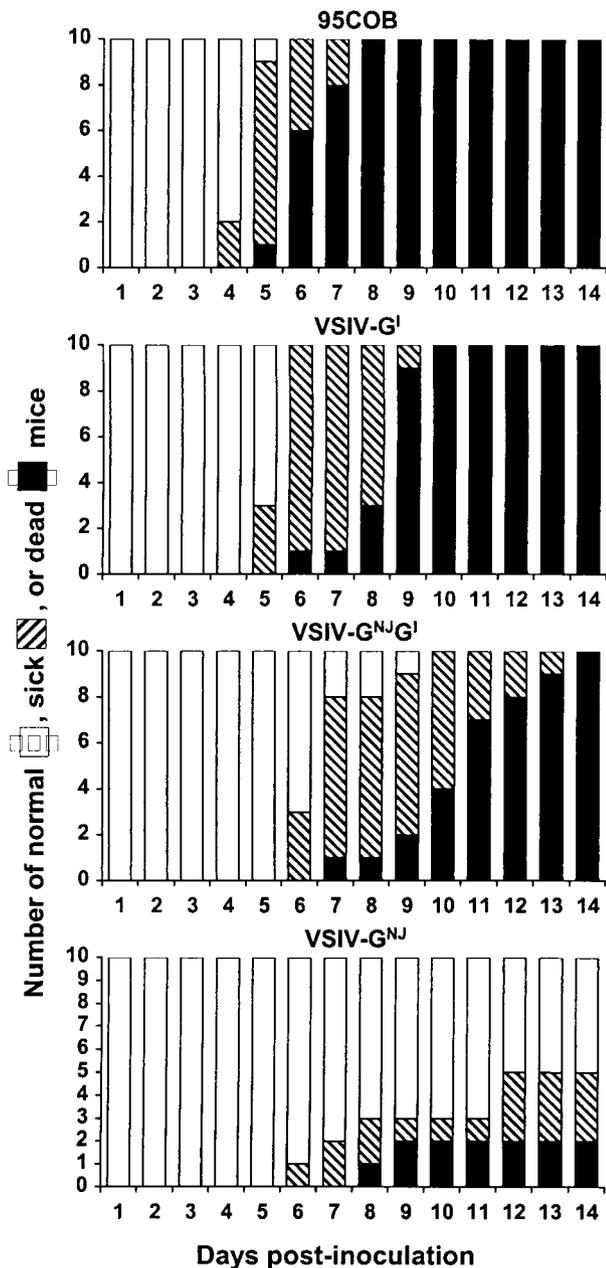


FIG. 4. Lethalities of wild-type and recombinant viruses in mice. The viruses were inoculated intranasally into groups of 10 mice at a dose of 10³ PFU/mouse. The mice were monitored daily for signs of morbidity and mortality. No further changes occurred after day 14.

VSIV-G^{nj}G¹ and VSIV-G^{nj} compared to VSIV-G¹. Symptoms of illness started as early as 5 to 6 days postinfection with VSIV-G¹ and were usually followed by death 7 to 8 days postinfection (Fig. 4). The generalization of symptoms was typically delayed 1 to 2 days in VSIV-G^{nj}G¹, and the time between the onset of sickness and death was extended by 3 to 4 days compared to VSIV-G¹. Inoculation of VSIV-G^{nj} into mice rarely resulted in death, so the LD₅₀ could not be determined. In contrast, 95COB virus was highly pathogenic in mice when inoculated intranasally (LD₅₀ = 16 PFU/mouse) (Fig. 4). Al-

though the replacement of G¹ by G^{nj} was responsible for the highly attenuated phenotype of VSIV-G^{nj}, this effect was compensated for in the 95COB virus. Since VSIV-G^{nj} and 95COB differ in genes other than the G gene, those genes may contribute to the different pathogenicities in the mouse model.

Pathogenesis in swine. Previous experiments showed important differences in pathogenicity between VSNJV and VSIV strains when pigs were inoculated experimentally by scarification of the snout, a procedure that leads to development of clinical symptoms and a disease course that closely resembles natural infection (14, 31, 51). VSNJV 95COB is a highly pathogenic field isolate that causes much more severe disease than recombinant or wild-type VSIV (14; L. L. Rodriguez, unpublished results). To test the hypothesis that the G glycoprotein was involved in the differential pathogenesis of VSIV and VSNJV, groups of four (VSIV-G¹ and 95COB) or eight (VSIV-G^{nj} and VSIV-G^{nj}G¹) Yorkshire pigs (8 to 10 weeks old and weighing 25 to 30 kg), which were negative for VSIV and VSNJV as indicated by serum neutralizing assay, were inoculated intradermally by scarification of the snout with 3 × 10⁶ PFU per animal as previously described (14). Each group was kept in a different room under BL-3 conditions at Plum Island Animal Disease Center and monitored daily for vesicular lesions and for rectal temperature. The extents of disease resulting from inoculation with the different viruses were evaluated by assessing lesion formation using a clinical scoring system based on the size and location of the resulting lesions (see Materials and Methods).

In general, small vesicles (<2 cm in diameter) developed the second day postinoculation at the area of scarification in most of the pigs. In severe cases, these vesicles increased in size to >2 cm and ruptured by days 3 to 5. At 6 to 7 days postinoculation, the lesions started to heal. Important differences, depending on the virus inoculated, were observed in the numbers of pigs that developed vesicles, the sizes and extents of the vesicular lesions, spread to distal areas, and time of appearance and healing of the lesions. The clinical scores from the snout of each pig inoculated with one of the four different viruses are shown for the 7 days following inoculation in Fig. 5B.

A progressive increase in the severity of the lesions was observed in inoculated pigs in the following order: VSIV-G¹ < VSIV-G^{nj}G¹ < VSIV-G^{nj} < 95COB (Fig. 5A). All four pigs inoculated with 95COB developed snout vesicles >2 cm in diameter as early as 2 days postinoculation, and the lesions reached scores of 4 by days 4 to 5 (Fig. 5B). In addition, two swine developed severe secondary lesions at the coronary bands of the hooves. In contrast, pigs inoculated with VSIV-G¹ developed only mild lesions. One pig inoculated with VSIV-G¹ never developed lesions, only one developed snout vesicles >2 cm in diameter 5 days postinfection, and one animal showed a small secondary foot lesion. Six of eight animals inoculated with VSIV-G^{nj}G¹ developed lesions, two exhibited vesicles >2 cm in diameter, and one developed small lesions on the snout outside the area of inoculation. All pigs inoculated with VSIV-G^{nj} developed large vesicles at the inoculation site, and five developed lesions on the snout outside the area of scarification. These lesions were especially severe in two pigs, which received clinical scores of 4. Compared to 95COB, a delay of ~2 days in vesicle development was observed in pigs inoculated with VSIV-G^{nj}, with vesicles reaching maximum severity

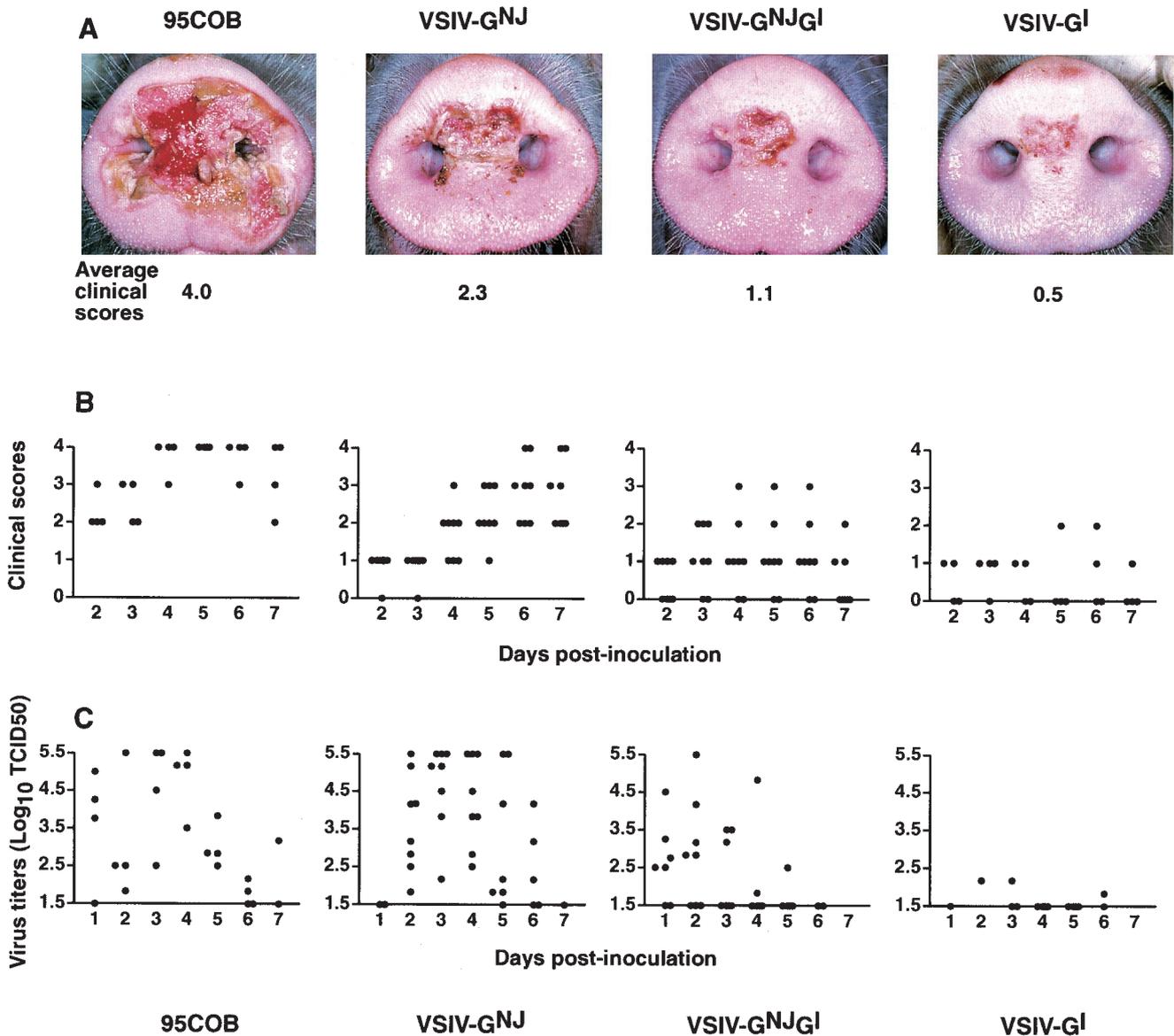


FIG. 5. (A) Snouts of pigs inoculated 5 days before with recombinant and wild-type VSVs. The swine were anesthetized, and 3×10^6 PFU of the indicated virus was applied by scarification of the snout. The snouts of the most severely affected animals from each group are represented. The average clinical scores from the snouts of all animals from each group 5 days postinfection are also shown. (B) Clinical scores after inoculation of pigs with the recombinant and wild-type viruses. The clinical scores refer to the snout and were based on the sizes and locations of viral lesions (see the text). Each symbol represents an individual animal. (C) Titers of virus recovered following inoculation with recombinant and wild-type viruses. Nasal swabs and EPF were taken daily from inoculated swine. Virus was recovered and titrated on BHK-21 cells at 35.5°C for 3 days. Each symbol represents an individual animal and corresponds to the highest titer obtained from the nasal swab or EPF. The virus dilution giving a titer of $5.5 \log_{10}$ was the highest dilution assayed, and samples to which this value was assigned might actually have higher titers of virus. TCID₅₀, 50% tissue culture infectious dose.

by days 6 to 7 postinoculation (Fig. 5B). Neither VSIV-G^{NJ}G^I nor VSIV-G^{NJ} caused secondary lesions on the feet. The pathological changes primarily involved the epidermis during the early stages but extended down to subcutaneous tissues in 95COB- and VSIV-G^{NJ}-infected pigs, causing loss of epithelium and leaving a bleeding surface in the most severe cases (Fig. 5A). This process was less apparent in lesions caused by VSIV-G^I. In agreement with previous results (14, 20, 52), we were unable to correlate rectal temperature with the severity of the infection.

As might be expected, none of the recombinant viruses were as virulent as the wild-type VSIV 95COB. This virus is a field

isolate with low passage in tissue culture, and viral products other than G may contribute to its increased pathogenicity. It may also be important that G^{NJ} is expressed at higher levels in 95COB than in the recombinant viruses and/or that the G^{NJ} protein in the engineered viruses might not interact optimally with other VSIV proteins (e.g., M), and this might lead to suboptimal replication and/or assembly of the recombinant viruses containing G^{NJ}. Nevertheless, the results presented above showed that the replacement of the G^I protein by the G^{NJ} protein from 95COB alone was able to increase the pathogenicity of VSIV in pigs.

Virus replication in swine. To investigate the ability of inoculated viruses to replicate in pigs, nasal swabs and EPF samples were taken on days 1 to 7 postinfection and clarified by centrifugation. Viruses were titered on BHK-21 cell monolayers in 96-well plates by serially diluting the supernatants and infecting cells in quadruplicate wells. The cells were incubated at 35.5°C for 3 days, and the 50% tissue culture infective dose was calculated. All animals were negative for virus isolation on the day before inoculation. In all groups, virus was isolated as early as 1 day postinfection from nasal swabs and 2 days postinfection from EPF. Typically, recovery from nasal swabs was more consistent and the titers were higher than those from EPF.

In agreement with the clinical scores shown in Fig. 5B, the virus titers recovered from pigs inoculated with 95COB and VSIV-G^{NJ} were generally highest, followed by VSIV-G^{NJ}G^I and then by VSIV-G^I (Fig. 5C). Within each group, the pigs with higher titers of virus also developed more severe lesions. These results suggest that VSV pathology in swine correlates primarily with virus replication. Differences in virus titers of up to 10,000-fold were detected between pigs inoculated with VSIV-G^{NJ} and those inoculated with VSIV-G^I, confirming a role for the G^{NJ} glycoprotein in the replication and pathogenicity of VSV in swine. The increase in the titers of viruses isolated from the VSIV-G^{NJ} group was delayed 1 day compared to those from 95COB and VSIV-G^{NJ}G^I, as seen from the clinical scores (Fig. 5B and C). There were also differences in the average lengths of time the virus was shed among the groups of pigs inoculated with different viruses. VSIV-G^I was shed for the shortest period (2.7 ± 1.7 days), followed by VSIV-G^{NJ}G^I (3.9 ± 0.9 days), VSIV-G^{NJ} (4.1 ± 0.8 days), and 95COB (5.7 ± 0.9 days) ($P < 0.001$).

DISCUSSION

The purpose of this work was to begin elucidating the basis of the marked differences in virulence and field occurrence observed between the VSNJV and VSIV serotypes. In this study, we generated recombinant cDNAs from which we recovered VSIVs that expressed one or both of the glycoproteins from the two major VSV serotypes. These recombinant viruses allowed us to explore the possibility of expressing VSIV and VSNJV glycoproteins from a single virus and the roles of these glycoproteins in virus replication, gene expression, and pathogenesis in laboratory animals and natural hosts.

All the engineered viruses were viable and synthesized the appropriate proteins. Neither the insertion of an additional gene (VSIV-G^{NJ}G^I) nor the replacement of the homologous G^I gene by the heterologous G^{NJ} gene (VSIV-G^{NJ}) had any detectable effect on the abilities of the recombinant viruses to replicate in BHK-21 cells, as assayed by single-step growth curves.

Although all the recombinant viruses grew equally well in BHK-21 cells, VSIV-G^{NJ}G^I and VSIV-G^{NJ} were attenuated in mice. Primary viral infections in animals are often a race, with viral replication and spread versus innate immune response and virus clearance. In this scenario, even small impairments in virus replication and/or spread, not detectable in single-step growth curves *in vitro*, could become critical for pathogenesis in mice (42, 57). The insertion of an additional heterologous

gene and subsequent downregulation of the downstream G^I and L genes may account for the moderately attenuated phenotype of VSIV-G^{NJ}G^I. We do not know the cause(s) of the highly attenuated phenotype of VSIV-G^{NJ} in mice, but several effects, alone or in combination, may have an influence in modulating the pathogenesis of this virus. For example, the lower expression of G^{NJ} and/or the suboptimal interaction of G^{NJ} with other viral protein(s) from the Indiana serotype during VSIV-G^{NJ} infection may be responsible. We are working to test these possibilities. A recombinant VSIV carrying G^{NJ} has been previously reported to grow to lower titers in tissue culture than the parental virus and to be attenuated in mice (28, 47). However, substantial differences exist between the extents of pathogenesis observed in mice for the parental recombinant VSIV used in those studies and the VSIV used here that preclude direct comparison between the VSIV-G^{NJ} viruses (references 15, 41, and 57 and this study).

Although the pathogenesis of VSV New Jersey and Indiana infection in laboratory rodents has been extensively examined, similar studies have not been performed in the natural hosts, and the molecular mechanisms involved remain largely unresolved. We have made use of a domestic-swine model (14) to study the pathogenesis of VSV. Inoculation by scarification of the snout led to the development of clinical symptoms and a disease course that closely resembled that observed in nature (31). Following the experimental inoculation of pigs, we observed that vesicles formed at the site of inoculation and ruptured, leaving reddish denuded erosions with exfoliated tissue adhering to the margins of the lesions. The erosive stage lasted for a week or so, and virus could be isolated from nasal swabs and EPF for 6 to 7 days after inoculation. Although pathological changes were limited to the epithelia of affected areas in most of the pigs, secondary lesions developed in the hooves of some animals. Depression, lameness, and excessive salivation were also seen. In this animal model, we observed that VSIV-G^{NJ} replicated to higher titers and caused more severe lesions than the parental VSIV-G^I. These results identified the G glycoprotein as an important determinant for VSV pathogenicity in swine, a natural host for VSV. Data from the recombinant virus expressing glycoproteins from two serotypes (VSIV-G^{NJ}G^I) further supported this conclusion. VSIV-G^{NJ}G^I was more pathogenic than VSIV-G^I, despite the fact that G^{NJ} represented only 40% of the total G protein incorporated into VSIV-G^{NJ}G^I virions. These results also illustrate the profound differences between mice and swine in VSV pathogenesis and emphasize the importance of the study of virus-host interactions in the natural hosts whenever possible.

Several studies indicate that the G protein plays an essential role in the pathogenesis of other viruses, including the rhabdoviruses rabies virus and viral hemorrhagic septicemia virus (12, 18, 22, 49, 53). The entry half-time of pathogenic rabies viruses in neuroblastoma cells was seven to eight times shorter than that of nonpathogenic variants (11). In addition, nonpathogenic viruses were unable to spread from cell to cell and displayed a lower rate of spread through the central nervous system. Other studies showed that pathogenic viruses apparently could use receptors or routes of entry different from those used by their nonpathogenic derivatives (10, 26). Fusion of rabies virus-infected cells may also be an important pathogenic mechanism (37). Changes in the surface envelope gly-

coprotein of simian-human immunodeficiency virus molecular clones have also been reported to be responsible for increases in fusion capacity, cytopathicity, replication capacity, and neutralization resistance (8).

In order to elucidate the mechanisms responsible for the differences in pathogenicity between VSV-G^{IN} and VSV-G^{NJ} viruses, functional analyses comparing the G proteins from the two serotypes similar to those described above for rabies virus and simian-human immunodeficiency virus are needed. Reverse genetic techniques for manipulating viral genomes and the domestic-swine model for VSV infection described here and elsewhere (14) constitute powerful tools for studies exploring the roles of individual proteins in the life cycle of this pathogen and for vaccine development.

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