Molecular Analysis of Neurovirulent Strains of Sindbis Virus That Evolve during Persistent Infection of scid Mice

BETH LEVINE† AND DIANE E. GRIFFIN1,2

Departments of Medicine1 and Neurology,2 Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

Received 21 June 1993/accepted 30 July 1993

To understand the role of tissue-specific adaptation and antibody-induced selectional pressures in the evolution of neurovirulent viruses, we analyzed three strains of Sindbis virus isolated from the brains of persistently infected scid mice and four strains of Sindbis virus isolated from the brains of scid mice with viral reactivation following immune serum treatment. For each viral isolate, we tested neurovirulence in weaning BALB/c mice and sequenced regions of the E2 and E1 envelope glycoprotein genes that are known to contain important determinants of Sindbis virus neurovirulence. One strain isolated from a persistently infected scid mouse and two strains isolated from scid mice with viral reactivation were neurovirulent, resulting in mortality in 80 to 100% of weaning BALB/c mice. All three neurovirulent strains contained an A→U change at nucleotide 8795, which predicts a Gln→His substitution at E2 amino acid position 55. No nucleotide changes were detected in the other sequenced regions of the E2 and E1 envelope glycoprotein genes or in the avirulent isolates. Our findings indicate that tissue-specific adaptations, rather than antibody-induced selectional pressures, are a critical determinant of the evolution of neurovirulent strains of Sindbis virus and provide evidence that E2 His-55 is an important neuroadaptive mutation that confers neurovirulence properties on Sindbis virus.

The effect of natural selection on viral virulence is critical to understanding the pathogenesis of persistent viral infections. For example, a central unresolved question in human immunodeficiency virus pathogenesis is whether the sequence divergence that occurs among human immunodeficiency virus strains isolated from individual patients over time (8, 20) plays a role in disease progression. Previous studies have demonstrated a role for neutralizing antibody in the selection of viral variants during persistent lentivirus infections (3) and a role for tissue factors in the selection of viral variants during persistent lymphocytic choriomeningitis virus (1, 2) and human immunodeficiency virus infection (9, 15). However, the relationships between immunologic and organ-specific selection in the emergence of viral variants with increased host virulence is not well understood.

To investigate the role of tissue-specific and antibody-induced selectional pressures in the evolution of viral mutants with increased neurovirulence, we analyzed mutant strains of Sindbis virus (SV) that evolve during persistent infection of antibody-treated and untreated severe combined immune-deficient (scid) mice. Previously, we have shown that scid mice develop persistent productive SV infection that can be restricted by treatment with SV hyperimmune serum (HIS) or monoclonal antibodies to the E2 envelope glycoprotein (11). Between 30 and 90 days after treatment with SV HIS, viral reactivation occurs in 30 to 60% of scid mice (10). Therefore, this model can be used to compare mutant strains of SV that arise in vivo in the absence of any cellular or humoral immune responses with those that arise in the presence of immune serum therapy. In addition, scid/CB17 mice are not susceptible to fatal encephalitis caused by NSV (31), a strain of SV that is neurovirulent in weaning BALB/c mice (6), suggesting that persistently infected scid mice may be able to harbor neurovirulent mutant strains of SV that would kill immunocompetent mice. Consequently, this model provides a unique opportunity to study the in vivo interactions of neuroadaptation, antibody-induced selection, and the evolution of neurovirulent phenotypes of SV.

SV, the prototype alphavirus, is a single-stranded RNA virus of plus polarity that has a genome of 11,703 nucleotides. The 5’ two-thirds encodes the four nonstructural proteins, nsP1, nsP2, nsP3, and nsP4, which serve as the viral replicase and transcriptase (23). The 3’ third encodes the structural proteins, which are translated from a 26S subgenomic mRNA as a polyprotein that is cleaved posttranslationally to form five polypeptides—capsid, E3, E2, 6K, and E1. Three of these polypeptides, capsid protein and the envelope glycoproteins E1 and E2, are found on the mature SV virion. E1 and E2 are transmembrane proteins that associate with each other soon after synthesis to form stable, noncovalently linked heterodimers (19). The E1-E2 heterodimer is responsible for virus attachment to the cell surface (E2) (28, 30) and mediates acid-dependent fusion of the virion with the endosomal membrane (E1) (18). Both E1 and E2 elicit neutralizing and protective antibody responses (5), but only anti-E2 monoclonal antibodies inhibit viral replication (11) and elicit anti-idiotypic antibodies that identify cellular receptors (28, 30).

Studies of recombinant strains of SV that differ in limited amino acid positions have provided important information about the molecular pathogenesis of SV encephalitis. The prototype strain AR339 causes fatal encephalitis in neonatal mice (24) and clinically silent encephalitis in weaning mice (25). Sequencing, construction of recombinant viruses, and site-directed mutagenesis studies have identified nucleotide positions in E1 and E2 envelope glycoprotein genes that are important determinants of virulence in suckling mice (4, 13, 14, 16, 17, 26). Important clues regarding the molecular basis of neurovirulence for weaning mice have been provided by cloning and sequencing the structural region of NSV, a strain of SV that causes 100% mortality in adult BALB/c mice. NSV

† Present address: Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032.

* Corresponding author.
was derived by serial intracerebral (i.c.) passage of AR339 (6) and differs from AR339 at two amino acid positions in the E2 envelope glycoprotein (Gln-55→His and Arg-209→Gly) and two amino acid positions in the E1 envelope glycoprotein (Val-72→Ala and Gly-313→Asp) (13). Of these four changes, only the histidine substitution at position 55 of E2 has thus far been shown to be a determinant of neurovirulence in weanling mice (5, 27).

To study the interactions between neuroadaptation, antibody-induced selection of antigenic variants, and the evolution of SV strains with increased neurovirulence, we screened whole-brain homogenates from scid mice at serial time points after SV infection for the presence of neurovirulent mutants. Four- to six-week-old scid/CB17 mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) were inoculated i.c. with $10^3$ PFU of SV strain AR339 on day 0. Persistently infected scid/CB17 mice were sacrificed at 6, 10, 20, and 30 days after SV infection (three mice per time point). In addition, another group of scid/CB17 mice were treated with 0.2 cm$^3$ of SV HIS on day 7 after SV infection and sacrificed on days 37, 52, 67, and 97 after infection (days 30, 45, 60, and 90 after SV HIS treatment) (three to six mice per group). As previously reported, infectious virus was recovered from all scid/CB17 mice that had not received SV HIS (11) and from 40% of mice that had been treated with SV HIS 30 or more days prior to sacrifice (10). We infected 4- to 6-week-old BALB/cJ mice (5 to 10 mice per group) (Jackson Laboratories, Bar Harbor, Maine) i.c. with $10^3$ PFU of whole-brain homogenate from the 12 persistently infected scid/CB17 mice and from the 6 scid/CB17 mice with viral reactivation following SV HIS therapy. Mortality was determined by daily observation of the mice for 3 weeks after infection.

As shown in Fig. 1, brain homogenates recovered from persistently infected scid/CB17 mice within 20 days after SV infection caused no mortality (or clinically apparent disease) when reinoculated into immunocompetent 4- to 6-week-old BALB/cJ mice. However, two of three brain homogenates from scid/CB17 mice 30 days after SV infection were neurovirulent in BALB/cJ mice: 30A caused mortality in 60% of BALB/cJ mice, and 30B caused mortality in 40% of BALB/cJ mice. Brain homogenates from two of six scid mice with central nervous system reactivation (30 or more days after SV HIS treatment) (37A and 52A) caused 100% mortality in adult BALB/cJ mice, while homogenates from the other four mice were avirulent. These observations suggest that a critical duration of replication in mouse brain (>20 days) is required for the selection of mutants that have increased neurovirulence. They also suggest that the acquisition of a neurovirulent phenotype is not related to SV HIS therapy or the mechanism of SV reactivation, since neurovirulent mutants occurred in the absence of immunotherapy and the majority of viral reactivation strains were not neurovirulent.

To further characterize neurovirulent mutant strains of SV, we plaque purified viral strains from brain homogenates 30A, 30B, 30C, 37A, 37B, 52A, and 67B. This group was chosen specifically to include all those that produced fatal encephalitis in BALB/cJ mice (30A, 30B, 37A, and 52A) as well as control avirulent brain homogenates harvested from mice at similar time points after SV infection. The plaque-purified strains of avirulent brain homogenates were also avirulent, and the mortality caused by the plaque-purified isolate from 52A was similar to that caused by the original brain homogenate (Fig. 1). For the other three neurovirulent strains (30A, 30B, and 37A), the mortality differed somewhat between the whole-brain homogenate and the plaque-purified isolate, reflecting a heterogeneous population of viral strains in the brain homogenate.

To gain insight into the molecular basis of the evolution of neurovirulent phenotypes during persistent infection of the mouse brain, the regions of the E2 and E1 envelope glycoprotein genes that differ between AR339 and NSV were sequenced in all seven plaque-purified isolates (30A, 30B, 30C, 37A, 37B, 52A, and 67B). The regions sequenced encode amino acids 1 to 87 and 128 to 216 of E2 and 39 to 148 and 220 to 334 of E1. To prepare viral cDNA for sequencing, we infected $10^6$ BHK cells with each viral isolate (multiplicity of infection of 0.1 PFU per cell) and harvested total RNA at 4 h after infection, using the RNAzol method (Tel-Test, Inc., Friendswood, Tex.). Viral cDNA was synthesized with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described previously (10) with the exception that oligo(dT) primers were used. Polymerase chain reaction amplification of the two regions of E2 and the two regions of E1 was performed using the following sets of primers: E2 plus-strand (nucleotides 8571 to 8590) 5'-GCCTACGATACTCTGCTCAA-3' and minus-strand (nucleotides 8893 to 8912) 5'-AAAGCCTTCTCACGGTCCGT-3', E2 plus-strand (nucleotides 8991 to 9010) 5'-GCAACGTCTAGTCTACCTGGC-3' and minus-strand (nucleotides 9279 to 9298) 5'-ATGGCGGGTCAACCCAGTGT-3', E1 plus-strand (nucleotides 10159 to 10178) 5'-CTGTACGTCCTGGAGAT-3' and minus-strand (nucleotides 10509 to 10528) 5'-CTACTGCTGGTACCTCGT-3', and E1 plus-strand (nucleotides 10701 to 10720) 5'-CTACGCGCCACGAGCAGACAT-3' and minus-strand (nucleotides 11065 to 11084) 5'-TTGGAGATTGCTGTTGCTCTG-3'. Polymerase chain reaction conditions were identical to those previously described (10) except that 35 cycles of amplification were performed. Polymerase chain reaction products were visualized on an agarose gel, purified by electrophoresis, and sequenced by the dideoxy chain termination sequencing method of Sanger et al. (21).
FIG. 2. Relationship between amino acid sequences of envelope glycoproteins and neurovirulence among SV strains isolated from persistently infected scid mouse brain. The amino acids that differ between wild-type SV AR339 and neuroadapted NSV are shown. The amino acid located at each of these positions is included for each virus strain isolated from persistently infected scid/CB17 mouse brain. 

The results of the sequencing are summarized in Fig. 2. The amino acid positions that differ in NSV and AR339 are shown at the top of the figure. The predicted amino acid sequence at these four positions is shown for the seven isolates sequenced. The only nucleotide change detected in any of the seven mutants was an A→G change at nucleotide 8795, which predicts a Gln→His change at E2 amino acid position 55. The presence of a predicted histidine at position 55 correlated with the ability to cause fatal SV encephalitis in adult BALB/cJ mice. Three isolates had histidine at position 55; of these, two isolates (30A and 52A) caused 100% mortality and one isolate (37A) caused 80% mortality. The four strains that did not have the change to histidine were avirulent, with the exception of 30B, which had a 20% mortality rate. No other nucleotide changes were detected in the regions of E1 and E2 of 30B that were sequenced, but this isolate may have mutations elsewhere in the genome that cause a partial increase in virulence.

The absence of any unique changes in viral isolates from scid mice treated with SV HIS compared with viral isolates from persistently infected scid mice was an unexpected finding. Several important antigenic determinants are located in the region of E2 between amino acids 181 and 210 (which encompass a glycosylation site at residue 196), as evidenced by the presence of specific amino acid changes in anti-E2 monoclonal antibody-neutralizing escape mutants (22). The conservation of primary structure in this region suggests that antigenic variants were not specifically selected for by the single treatment with SV HIS. Furthermore, enzyme-linked immunosorbent assay reactivities with SV HIS do not differ significantly between neurovirulent mutants (that arose during persistent infection or reactivated infection after SV HIS therapy) and wild-type AR339 (data not shown). In addition, a previous study demonstrated that repeat treatment with SV HIS results in the clearance of neurovirulent isolate 52A from persistently infected scid/CB17 mouse brain, indicating that this strain is not an antibody escape mutant (10). Taken together, these observations indicate that antibody-induced selection pressures do not play an important role in vivo in the evolution of neurovirulent SV phenotypes in this study.

Our findings indicate that the process of neuroadaptation that occurs during prolonged replication in the mouse brain is a critical factor in the emergence of SV mutant strains that have increased neurovirulence in adult immunocompetent mice. Our observation that a histidine substitution at E2 position 55 is strongly correlated with the ability to cause fatal disease in adult mice provides important information about the molecular basis of tissue-specific adaptation and neurovirulence. Although other changes in regions of the SV genome that were not sequenced in the neurovirulent mutants may be present, construction of recombinant viruses and site-directed mutagenesis studies of amino acid position 55 of E2 have already confirmed the importance of a single histidine substitution at position 55 in neurovirulence for older mice (27).

Several mechanisms can be proposed to explain how a histidine substitution at E2 position 55 contributes to the neurovirulence of SV in adult mice. Viruses with His-55 grow more rapidly and to higher titers in the brains of 7- and 14-day-old mice, suggesting that His-55 may increase neurovirulence in older mice by increasing the efficiency of virus replication (27). Additionally, a histidine substitution at E2 position 55 specifically blocks the protective effect of the cellular oncogene bcl-2 on SV-induced cell death in vitro (29). A similar mechanism may be important in vivo, since inhibition of apoptosis in the mature nervous system has been postulated to play a role in the protection of adult mice against fatal SV infection (7, 12). Finally, a histidine substitution at E2 position 55 may either elicit or mediate important immunopathologic host responses, since only immunocompetent and not scid mice develop fatal encephalitis after infection with strains containing this mutation (31).

Thus, a single amino acid change in the SV E2 envelope glycoprotein that evolves during persistent infection of mouse brain increases viral growth (27), counteracts the protective effects of a host cell gene that is expressed in the nervous system (29), and correlates with the acquired ability to cause fatal disease in adult immunocompetent mice. These observations suggest that one point mutation selected for during tissue-specific adaptation may be sufficient to enable a virus to develop multiple strategies to increase its virulence potential.

We thank Marcia B. Lyons for excellent technical assistance and Roxann Ingersoll for sequencing of polymerase chain reaction products. This work was supported by research grant NS18596 (D.E.G.) and a training grant (T32-NS-07000) (B.L.) from the National Institutes of Health.
REFERENCES


31. Wesselingh, S., B. Levine, R. J. Fox, S. Choi, and D. E. Griffin. Intracerebral cytokine mRNA expression during fatal and nonfatal alphavirus encephalitis suggests a predominant type 2 T cell response. Submitted for publication.