Viral Determinants of Age-Dependent Virulence of Sindbis Virus for Mice

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Many alphaviruses cause more severe disease in young animals than in older animals. The age-dependent resistance to severe disease is determined primarily by maturation of the host, but strains of virus can be selected that overcome the increased resistance of mature animals. Sindbis virus (SV) strain AR339 causes fatal encephalitis in newborn mice and nonfatal encephalitis in weanling mice, whereas NSV, a neuroadapted strain of SV, causes fatal encephalitis in weanling as well as newborn mice. We have previously shown that the E2 glycoprotein of NSV contained His-55, whereas AR339 E2 had Glu-55 (S. Lustig, A. C. Jackson, C. S. Hahn, D. E. Griffin, E. G. Strauss, and J. H. Strauss, J. Virol. 62:2329–2336, 1988) and that SV with E2 containing Gly-172 was more virulent for newborn mice than SV with E2 containing Arg-172 (P. C. Tucker and D. E. Griffin, J. Virol. 65:1551–1557, 1991). Here we tested the virulence for both newborn and older mice of SV containing a number of different amino acids at E2 position 55 (His, Glu, Lys, Arg, Glu, Gly) in combination with both Gly-172 and Arg-172. All the viruses were virulent for newborn mice, but the residues at both 55 and 172 influenced the virulence of the virus, and there were differences in virulence observed among the various viruses. However, only viruses with His-55 were fully virulent for 14-day-old mice, and this virulence was independent of the residue at position 172. Virus with Lys-55 was virulent for 7-day-old mice, although slightly attenuated relative to His-55. Viruses with His-55 grew more rapidly and to higher titer in the brains of 7- and 14-day-old mice, in N18 neuroblastoma cells, and in BHK cells. Our data suggest that His-55 is important for neurovirulence in older mice and acts by increasing the efficiency of virus replication.

In the New World, alphaviruses are important causes of arthropod-borne encephalitis in humans. The outcome of these infections is dependent on the age of the host at the time of infection and the virulence of the infecting virus. Sindbis virus (SV), the prototype alphavirus, causes age-dependent encephalitis in mice (12, 23) and provides an excellent animal model for studying the pathogenesis of alphavirus infection of the central nervous system. Studies of recombinant strains of SV that differ in a limited number of amino acids has proved useful in the identification of the molecular basis of the pathogenesis of alphavirus encephalitis (16, 21, 22, 31).

SV is a single-stranded RNA virus of plus polarity that has a genome of 11,703 nucleotides. The 5′ two-thirds of the genome encodes the nonstructural proteins that participate in RNA replication (30), and the 3′ one-third encodes the structural proteins, which consist of a capsid protein and two envelope glycoproteins. The surface glycoproteins E1 and E2 present in the virus envelope form a stable heterodimer (26) that acts as a functional subunit. Cell binding and penetration are probably functions of the heterodimer, since antibodies to both E1 and E2 can block hemagglutination and neutralize virus infectivity (27, 29). However, the most potent neutralizing antibodies bind to E2 and elicit anti-idiotypic antibodies that identify cellular receptors (32, 35). It is likely that E1, which is more highly conserved, carries the fusion activity by which the virus enters the cell (25) and also participates in cell attachment (3).

The prototype strain (AR339) of SV causes 100% mortality in suckling mice and 0% mortality in weanling mice (12, 23), while a more neurovirulent strain (NSV), recovered after several passages in mouse brain, can cause fatal infection in mice of all ages (9). Both viruses replicate in neurons but NSV grows to higher titer than AR339 in the brains of weanling mice and causes more severe neuronal damage (10, 11). Sequencing of the structural region genes has shown that these two viruses differ at two positions in the E2 glycoprotein (55 and 209) (16) (Fig. 1). The relative contribution of each substitution for increased virulence in older mice was not known, but it was postulated that the change from Glu (AR339) to His (NSV) at position 55 of the E2 glycoprotein is important for age-dependent virulence since this substitution is not seen in avirulent laboratory-adapted strains of SV (8, 16). In addition, a mutant of the HRNJ strain of SV also has His at E2 position 55 and is virulent for weanling mice (5, 8).

To determine more definitively the importance of His-55 for age-dependent changes in virulence, we constructed recombinant viruses differing only at this position and determined their virulence for mice of different ages. Since previous studies had shown that a change from Arg (HRSP) to Gly (AR339 and NSV) at E2 position 172 increases SV virulence in newborn mice (16, 31), recombinant viruses were also constructed to determine whether this substitution makes an independent contribution to virulence in older mice.
controls. Individual plaques were picked from the primary transfections and used to generate stocks in BHK cells.

**METHODS**

**Cell lines.** BHK-21 cells and the N18 clone (2) of C1300 mouse neuroblastoma cells (obtained from M. Nirenberg, National Institutes of Health) were grown in Dulbecco’s minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum and 50 µg of gentamicin per ml.

**Construction of viruses.** Two full-length cDNA clones, TE and TES, were previously constructed by replacing the genes for E1 and E2 in the full-length clone Toto1101 by the Plasmid E2 and El glycoproteins of the residues 172.

**Site-directed mutagenesis.** Site-directed mutagenesis was accomplished by the in vitro method of Kunkel (12a), using the degenerate oligonucleotide 8801-TCCGTT(CT)(CT)GTCGTAT-8786, and mutant bacteriophages were screened by sequencing in the region of the expected mutation. NcoI-Stul fragments (297 nt as described above) of the mutagenized M13 RFs were reintegrated into the full-length Sindbis clones by a three-piece ligation with the Stul-Sac1 (8.8-kgb) and SacI-NcoI (4.7-kb) fragments of either E2(H-55G-172) (for constructs with G at position 172 of E2) or E2(H-55S-172) (for constructs with R at position 172). In each construct, the entire NcoI-Stul insert was sequenced directly from double-stranded plasmid DNA of the full-length clones, and more than one correct construct for each mutation was examined for infectivity by transfection and transfection on BHK cells. In each case, the number of plaques produced in the primary transfection assay was comparable to wild-type controls. Individual plaques were picked from the primary transfections and used to generate stocks in BHK cells.

**RESULTS**

**Effect of the amino acid at E2 position 55 upon virulence in mice.** To test the importance of His-55 in E2 for neurovirulence in mice, we constructed recombinant SVs that had either His-55 (the residue found in NSV, a virus neurovirulent for weanling mice) or Gln-55 (the residue found in AR339 SV, a virus neurovirulent for newborn mice but not for weanling mice) combined with both Gly-172 and Arg-172. The genomes of these viruses are illustrated schematically in Fig. 1 together with the genomes of various strains of SV that have been previously studied; the four recombinant viruses are identical except for the residues at positions 55 and 172 of E2. All four recombinant viruses caused essentially 100% mortality in 1-day-old mice when inoculated either i.c. or s.c. (Table 1). However, mice infected by viruses with His-55 have a shorter survival time than those

### TABLE 1. Virulence of recombinant viruses for newborn (1- to 2-day-old) mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>i.c.</th>
<th>s.c.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Survival</td>
<td>MDOD (SD)</td>
</tr>
<tr>
<td></td>
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<tr>
<td>E2(H-55G-172)</td>
<td>0/30</td>
<td>2.1 (±1.49)</td>
</tr>
<tr>
<td>E2(Q-55G-172)</td>
<td>0/39</td>
<td>3.9 (±1.49)</td>
</tr>
<tr>
<td>E2(H-55S-172)</td>
<td>0/28</td>
<td>2.6 (±0.77)</td>
</tr>
<tr>
<td>E2(Q-55S-172)</td>
<td>0/27</td>
<td>3.5 (±0.93)</td>
</tr>
</tbody>
</table>

* MDOD, mean day of death.
  
* NS, not significant.
with Gln-55. The mean day of death after i.c. infection by E2(H-55G-172) or E2(H-55R-172) was earlier than after infection by E2(Q-55G-172) or E2(Q-55R-172), and the mean day of death after s.c. infection by E2(H-55G-172) or E2(H-55R-172) was slightly earlier than after infection by E2(Q-55R-172). Extended survival times for mice after infection by viruses with Gln-55 was seen following i.c. inoculation, and we conclude that viruses with His-55 are somewhat more virulent for newborn mice than those with Gln-55. The survival time of the mice is also influenced by the residue at position 172 of SV E2. Mice infected by E2(H-55G-172) had the shortest survival time by either route of inoculation, and in general, survival time was extended by substitution of arginine at 172.

When the four viruses were tested in older mice, mortality remained high after infection by viruses containing His-55, but not after infection with viruses containing Gln-55 (Fig. 2). Therefore, the amino acid at position 172 had only minor effects at these older ages. In 7-day-old mice, E2(H-55G-172) caused 100% mortality when inoculated i.c. and 90% mortality when inoculated s.c., and E2(H-55R-172) caused 100% mortality i.c. and 94% s.c. In contrast, E2(Q-55G-172) caused only 18% mortality i.c. and 3% s.c., while E2(Q-55R-172) caused 18% mortality i.c. and 0% s.c. A similar pattern was also evident when the viruses were tested in 14-day-old mice. Both viruses with His at E2 position 55 were virulent [92% mortality i.c., 35% s.c. for E2(H-55G-172), and 88% mortality i.c., 38% s.c. for E2(H-55R-172)], while viruses with Gln at E2 position 55 were avirulent [0% mortality i.c. or s.c. for both E2(Q-55G-172) and E2(Q-55R-172)]. These data indicate that the amino acid at position 55 of E2 is an important determinant of neurovirulence in older mice.

To further explore the importance of the residue at E2 position 55, we site specifically changed this residue to Lys, Arg, Glu, or Gly. The virulence of the resulting viruses, containing either Gly-172 or Arg-172 were tested in newborn and in weanling mice (Fig. 3). All the viruses were virulent for newborn mice, with relatively slight differences observed for the various viruses. However, the mutant viruses were attenuated in 7- and 14-day-old mice, although E2(K-55G-172), which retained a positive charge at E2 position 55, led to 65% mortality when given i.c. to 7-day-old mice. Thus, the amino acid residues tested at position 55 of E2, His-55 results in virus having the highest virulence for 7- and 14-day-old mice.

Replication of viruses in mouse brain. Increased virulence of NSV for weaning mice is associated with increased viral replication in neurons (11). To determine whether the amino acid at E2 position 55 affected the efficiency of replication in the central nervous system, we determined the replication of the four recombinant viruses containing either His-55 or Gln-55 combined with both Gly-172 and Arg-172 in both 1-day-old and 7-day-old mice after i.c. inoculation (Fig. 4). In 1-day-old mice, the identity of the residue at position 172 seemed to be of the greatest importance, at least early after infection. Viruses with a glycine at E2 position 172, E2(H-55G-172) and E2(Q-55G-172), grew similarly early after infection, with production of new virus detectable by 4 h after infection. Viruses with Arg at E2 position 172, E2(H-55R-172) and E2(Q-55R-172), exhibited the previously described delay in replication (Fig. 4A) (31). However, by 24 h after infection, E2(H-55G-172) gave the highest titer, consistent with the fact that newborn mice infected with this virus have the shortest survival time, with the other three viruses giving equivalent titers. In contrast, in 7-day-old mice, the residue at position 55 was the dominant influence. Viruses with histidine at 55, E2(H-55G-172) and E2(H-55R-172), grew more rapidly and to a higher titer than the viruses with Gln-55 (Fig. 4B). E2(H-55G-172) displayed a significant increase in replication over E2(Q-55G-172), depending on the time of assay (P < 0.05 at 2, 4, 6, and 10 h). E2(H-55R-172) displayed an increase in replication over E2(Q-55R-172) (P < 0.05 at 2, 4, 6, 8, 10, and 24 h). These data indicate that His-55 in E2 is important for efficient viral replication in mature neurons. However, His-55 provides less advantage over Gln-55 for growth in immature neurons, especially early after infection, whereas substitutions at E2 position 172 affect early replication in immature neurons.

Replication of recombinant viruses in cultured cells. To determine whether these alterations in replication in mouse brain were reflected in the growth rates in cultured cells, we examined replication of the four recombinant viruses in N18 cells, a mouse neuroblastoma cell line, and in BHK cells, a nonneuronal hamster kidney cell line. The identity of the residues at both positions 55 and 172 affected the growth rate of the virus. In N18 cells, virus E2(H-55G-172) grew most
rapidly, with significantly higher yields at 6 and 10 h than any of the other viruses ($P < 0.05$). \textit{E2(H-55R-172)} was significantly delayed in its growth, but by 24 h reached titers greater than \textit{E2(Q-55R-172)} ($P < 0.05$). \textit{E2(Q-55G-172)} grew slightly faster than \textit{E2(Q-55R-172)}, producing more virus at 2, 4, 6, 8, 10, and 24 h ($P < 0.05$). Thus, in terms of growth rate, viruses with Gly-172 have an advantage over those with \textit{Arg-172}, a pattern similar to the growth in 1-day-old mouse brains. Position 55 contributes to increased replication in N18 cells. For instance \textit{E2(H-55R-172)} exhibited more-rapid growth over \textit{E2(Q-55R-172)} ($P < 0.05$ at 2, 8, and 24 h) and \textit{E2(H-55G-172)} exhibited more-rapid growth than \textit{E2(Q-55G-172)} ($P < 0.05$ at 6, 8, and 10 h). A similar pattern was found in BHK cells, but the differences in growth rate were not as marked. Viruses with \textit{His-55} replicated similarly with significantly higher titers than virus with \textit{Gln-55} at 24 h ($P < 0.05$). \textit{E2(Q-55R-172)} replicated least well, with slower replication than virus with \textit{His-55} ($P < 0.05$ at 4, 6, 8, 10, and 24 h).

**DISCUSSION**

Immunologic factors have been proposed to account for age-dependent susceptibility to some viruses, but this does not appear to be a major factor in SV infection of mice. Replication of AR339 is limited in the brains of weanling mice in the absence of an immune response (12, 14, 18, 23), and susceptible 1-week-old mice develop cellular and humoral immune responses comparable to those of resistant older mice (7). Age-dependent susceptibility to SV encephalitis is dependent primarily on the ability of the virus to replicate in neurons at different stages of maturity and the effect of that replication on neurons. Neurons in immature mice are permissive for all but the most avirulent strains of SV (16, 28) and, with maturation, become progressively less permissive (12, 23). Adaptation in the virus can compensate for this age-dependent neuronal restriction of replication, resulting in viruses that are neurovirulent for progressively more mature mice. We showed that an important change leading to increased replication of SV in mature neurons is the substitution of \textit{His-55} for \textit{Gln-55} in the E2 glycoprotein. An adaptive advantage of the \textit{His-55} in E2 for growth in mature neurons is further suggested by the consistent appearance of \textit{His-55} in virus recovered from the brains of weanling mice months after infection with AR339 (which contains Gln-55) (13).

The surface glycoproteins of SV are known to be important determinants of virulence for mice (4, 16, 20–22, 31), but no particular amino acid change has previously been definitively shown to be associated with alterations in age-dependent virulence. A change from \textit{Ser} to \textit{Arg} at E2 position 114 resulted in decreased virulence for newborn mice and in more rapid penetration of BHK cells (4). Since a hydrophobicity plot places \textit{Ser} at E2 position 114 within a hydrophobic region, it was postulated that a change to \textit{Arg}...
may alter the conformation and/or stability of the E2 protein, but it is not known how this change decreases virulence for mice while increasing the rate of infection of BHK cells. It has also been shown that a change from Gly to Arg at E2 position 172 resulted in prolonged survival of newborn mice and decreased viral adsorption to neuronal cells (31), leading to the hypothesis that this region is important for binding to a neuronal receptor for SV.

His-55 in E2 could lead to increased susceptibility of older mice either by increasing the binding to and penetration of mature neurons by the virus or by increasing the efficiency of virus replication in mature neurons once the virus has initiated the infection. Although more studies are required, we suggest that the increased susceptibility arises, at least in part, from an increased efficiency of replication and perhaps increased cell damage in neurons by virus containing His-55. NSV consistently replicates to a higher titer than AR359 in the brain and spinal cord of weanling mice over the whole course of infection (11), and this increase in titer can be mimicked by the single change of His-55 to Gln-55 in E2 in a recombinant virus. Studies of Ross River virus, an alphavirus whose E2 glycoprotein shares 42% amino acid identity with SV E2, suggest that the region around E2 position 55 is important for the rate of viral replication (33). A mutant of the virulent T48 strain of the Ross River virus with a deletion of amino acids 55 to 61 of E2 (corresponding to residues 52 to 58 of SV E2) is less virulent for newborn mice but exhibits increased viral replication in BHK cells. It was postulated that a change in the conformation of E2 led to changes in replication in mice because the deletion mutant was more thermostable and had decreased reactivity with an E2-specific neutralizing monoclonal antibody (34), but effects on RNA replication may also be important. We are exploring the mechanisms by which His-55 might affect virulence by examining the binding and penetration of neuronal cells by different SV recombinants and by examining RNA synthesis following infection by an SV mutant with a deletion of E2 residues 52 to 58.

It is not clear how substitutions at E2 position 55 might affect RNA replication or other aspects of virus replication. The region around E2 position 55 is hydrophilic but has a low probability of being on the surface, and the substitution of His for Gln causes relatively little change in these predictions. The results of site-directed mutagenesis showed that none of the other amino acids tested (Lys, Arg, Glu, and Gly) resulted in a virus as virulent as when His is present. The possible importance of charge at this position is suggested by the fact that substitution of Lys did result in a virus that is moderately virulent for 1-week-old mice when given i.c.

The change in the host neuronal cell during maturation that normally restricts replication of SV is unknown. Multiple morphological and biochemical changes occur in the developing rodent brain after birth (6). Neural development proceeds through a sequence of cellular events that includes cell proliferation, migration, and differentiation and leads to establishment of synaptic connections. Proliferation of large neurons is completed prenatally, but cells destined to become the interconnecting small neurons continue to proliferate for 2 to 3 weeks after birth (1). Differentiating postmitotic neurons are actively involved in process outgrowth and synaptogenesis, which are associated with complex changes in gene expression (17) and in synthesis of different types of carbohydrates, proteins, and lipids. These changes include changes in viral receptors (32) which may contribute to the altered virulence associated with amino acid changes at E2 position 172 (31). Other changes in neurons that occur with maturation, such as an increased resistance to SV-induced apoptosis through expression of the bcl-2 oncogene (15), may also be important for limiting the amount of virus synthesized as well as altering the cellular outcome of infection. The specific change in neuronal metabolism that leads to decreased efficiency of replication of SV which can be overcome by substitution of His for Gln at E2 position 55 is not known. However, this maturational change may affect replication of other RNA viruses in neurons since age-dependent susceptibility to encephalitis is a characteristic of flaviviruses, enteroviruses, and bunyaviruses, as well as alphaviruses (19).

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