Mechanism of Altered Sindbis Virus Neurovirulence Associated with a Single-Amino-Acid Change in the E2 Glycoprotein

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The mechanism by which amino acid changes in the E1 and E2 surface glycoproteins of Sindbis virus affect neurovirulence is unknown. We have studied two recombinant viruses which differ in virulence. One (TE) contains Gly and the other (TES) contains Arg at position 172 in E2. TE causes more rapid death than TES in newborn mice. Both viruses replicate similarly in nonneuronal cells, but TE replicates more rapidly in the brains of newborn mice and in neuroblastoma cells. TE also induces earlier viral RNA synthesis in neuroblastoma cells. ³⁵S-labeled TE binds more efficiently to brain and neuroblastoma cells, but not to nonneuronal cells, than TES. We propose that a region of the E2 glycoprotein affected by the amino acid occupying position 172 is important for binding to an alphavirus receptor on neurons and influences neurovirulence by this mechanism.

Alphaviruses are important causes of arthropod-borne encephalitis. Sindbis virus, the prototype alphavirus, causes fever, rash, and arthritis or clinically silent disease in humans (7) but causes encephalitis in mice (15, 31). The virulence of Sindbis virus for mice is dependent on host age and virus strain. Infection with wild-type virus (strain AR339) results in fatal encephalitis in newborn mice and nonfatal encephalitis in weanling mice (15). A neuroadapted strain (NSV) isolated after several intracerebral passages is lethal to weanling mice if inoculated intracerebrally (i.c.) (10). Two other strains, HRSP (3) and Toto1101 (21), are less virulent for newborn mice than AR339 is (18, 26). Within the central nervous system, different strains of Sindbis virus replicate primarily in neurons (12, 26). Therefore, efficiency of replication in neurons is likely to be a primary determinant of virulence.

Sindbis virus has three major structural proteins in the mature virion, the capsid protein C and the envelope glycoproteins E1 and E2. E1 and E2 form a stable noncovalently linked heterodimer (16, 23) which is a functional subunit (2). Cell attachment is probably a function of the heterodimer, since monoclonal antibodies to both E1 and E2 can block hemagglutination and neutralize virus infectivity (25, 28). The highly conserved E1 glycoprotein carries the fusion activity by which the virus enters the cell (22), while the less highly conserved E2 glycoprotein elicits the most potent neutralizing antibodies.

Changes in the amino acid compositions of both the E1 and E2 glycoproteins result in altered virulence for mice (5, 18, 20), but to date the mechanism by which these alterations affect virulence has not been identified. To begin to determine the basis for glycoprotein-determined differences in neurovirulence, we have studied two recombinant strains of Sindbis virus, TE and TES. These recombinant viruses are identical except for position 172 of the E2 glycoprotein (Fig. 1). Both viruses have genes for the nonstructural and C proteins from Toto1101 and for the E1 glycoprotein from AR339. TE has the entire E2 gene from NSV, while TES has the N-terminal portion of E2 from NSV and the C-terminal

portion from HRSP, an avirulent strain. The only amino acid difference between NSV and HRSP in this region is at position 172. Therefore, at position 172 TE has Gly and TES has Arg (18).

Previous studies have shown that TE is more virulent for newborn and weanling mice than TES is, suggesting that the Arg at E2 position 172 results in decreased neurovirulence (18). To identify the mechanism for this alteration in virulence, we have compared the growth of TE and TES in the brains of newborn mice and in cells of neuronal and nonneuronal origin and have compared various steps in the virus life cycle in these different cell types. These studies have shown that decreased virulence is correlated with decreased binding of virus to cells of neural origin.

MATERIALS AND METHODS

Cell lines. The cell lines used for propagation, growth, RNA synthesis, and attachment assays were the baby hamster kidney cell line BHK-21, the mouse fibroblast cell line L-929, and clones NB41A3 (American Type Culture Collection, Rockville, Md.) and N18 (1; obtained from M. Nirenberg, National Institutes of Health) of mouse neuroblastoma cells. BHK-21 and N18 cells were grown in Dulbecco's minimal essential medium (GIBCO, Grand Island, N.Y.) plus 10% fetal bovine serum and 50 µg of gentamicin per ml. Mouse L-929 cells were grown in minimal essential medium (GIBCO) plus 10% fetal bovine serum and gentamicin. NB41A3 cells were grown in Ham's F10 medium (Flow Laboratories, McLean, Va.) plus 15% horse serum, 2.5% fetal bovine serum, and gentamicin.

Viruses. TE and TES were constructed in the laboratory of J. H. Strauss by using a full-length cDNA clone, Toto1101 (21), and restriction fragments from SV (AR339), NSV, and HRSP as previously described (18). The viruses had been plaque purified, and stocks were grown from the original plaqued stock and assayed in BHK-21 cells. Stock viruses contained 10⁸ to 10⁹ PFU/ml.

Animal experiments. One to three-day-old CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.; virus antibody free) were inoculated either i.c. in the left hemisphere or subcutaneously (s.c.) in each hind foot

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FIG. 1. Amino acid differences in the E2 glycoproteins of the strains of Sindbis virus relevant to this study. TE and TES differ only at position 172. NS, nonstructural region; C, capsid.

with 1,000 PFU of TE or TES per 0.03 ml of Hanks balanced salt solution containing 1% fetal bovine serum. Virus growth curves were determined by removing brains from three mice infected i.c. or hind feet and blood from three mice infected s.c. at various times after infection. The tissues were frozen and thawed, and 10% homogenates were prepared by using Hanks balanced salt solution as the diluent. Each homogenate was assayed for virus content by plaque formation in BHK-21 cells, and the geometric mean virus content was determined for each time point.

Immunohistochemistry. Brains taken 48 h after i.c. inoculation were fixed in buffered 4% paraformaldehyde at 4°C for 24 h, dehydrated, paraffin embedded, and sectioned. Deparaffinized sections were stained for Sindbis virus antigen as previously described (12) with an anti-Sindbis virus rabbit immunoglobulin G (IgG) and biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif.).

Growth in tissue culture. L-929 cells and BHK-21 cells were grown to confluency in 24-well tissue culture plates (Costar, Cambridge, Mass.). N18 cells were grown in wells pretreated for 2 h with 0.005 mM poly-DL-ornithine hydrobromide (Sigma Chemical Co., St. Louis, Mo.) in 7.5 mM borate buffer, pH 8.4. The cells were infected at a low multiplicity of infection (MOI) of 0.1 PFU per cell or a high MOI of 10 to 20 PFU per cell for 1 h, washed two times, and refed with 1 ml of Dulbecco's minimal essential medium plus 1% fetal bovine serum. Supernatant fluid was removed from three wells at each time point, and virus from each well was quantitated by plaque formation on BHK-21 cells.

Purification and radiolabeling of virus. BHK-21 cells were inoculated with each virus at an MOI of 1.0, incubated at 37°C for 1 h, and fed with methionine and cysteine-free minimal essential medium containing (per milliliter) 4.0 to 6.0 μ Ci of [35 S]methionine and 4.0 to 6.0 μ Ci of [35 S]cysteine (Amersham, Arlington Heights, Ill.). When >90% cytopathic effect was evident, the supernatant fluid was harvested and clarified, and the virus was precipitated in 10% (wt/vol) polyethylene glycol 8000 in 0.5 M NaCl for 1 to 2 h at 4°C. Virus was pelleted, resuspended in NET buffer (10 mM Tris, 3 mM EDTA, 150 mM NaCl, pH 7.3), and purified over a continuous gradient of 15 to 40% potassium tartrate in phosphate-buffered saline (PBS; pH 7.4) at 131,000 $\times$ g for 16 h. Visible bands were harvested and dialyzed overnight at 4°C against 0.05 M Tris, pH 7.4. Virus was diluted in binding medium (RPMI 1640 [without NaHCO_3], 0.2% bovine serum albumin, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.4 [8]) and stored at -70°C.

Radiolabeled preparations of TE and TES had a ratio of counts per minute to PFU of 0.0004 to 0.005.

Virus-binding assay. Brains from 3-day-old mice were minced, suspended in Hanks balanced salt solution, layered on Lympholyte-M (Accurate Chemical and Scientific Corp., Westbury, N.Y.), and spun at 600 $\times$ g for 20 min to remove erythrocytes, polymorphonuclear cells, dead cells, and debris. Neural cells were recovered from the interface for use in binding assays. N18 or NB41A3 cells (1×10^6) or mouse brain cells (1.5×10^5) were placed in 1.5-ml microcentrifuge tubes, washed twice with binding medium, and then suspended in 0.5 ml of binding medium precooled to 4°C. Virus was added at an MOI of 0.1 to 1.0 PFU per cell (at least 1,000 cpm per tube) according to the specific activity of the individual batch of virus, and the cell-virus mixture was continuously and gently rocked at 4°C to keep the cells in suspension. At various time points, the cells from duplicate tubes were washed twice with cold binding medium. The supernatant and wash fluids from each tube were pooled, and radioactivity was counted to determine unbound virus. The pelleted cells were resuspended in 1% sodium dodecyl sulfate (SDS; BDH Chemical Ltd., Poole, England) and counted to determine bound virus. Binding to BHK-21 cells was determined with confluent monolayers in 12-well plates. Virus was added at an MOI of 1.0 in 0.3 ml of binding medium and rocked at 4°C. At various time points, duplicate wells were washed twice with 0.3 ml of cold binding medium, and the supernatant and wash fluids were pooled to determine unbound virus. The cells were lysed with 1% SDS and counted to determine bound virus. Samples were suspended in Liquiscint (National Diagnostics, Manville, N.J.), and the radioactivity in the samples was counted in a liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

RNA synthesis. Monolayers of N18 cells were infected with TE or TES at an MOI of 10 to 15, and at hourly intervals the supernatant fluid was removed and the cells were lysed in GED (5 M guanidine isothiocyanate, 0.1 M EDTA [pH 7.0], 10 mM dithiothreitol) at a concentration of 1 ml/20 million cells (19). The lysed cells were placed in microcentrifuge tubes, vortexed vigorously for 1 to 2 min, and stored at -70°C. A pGEM plasmid containing DNA encoding the entire structural region of HRSP (gift of J. H. Strauss and E. Strauss) was used for solution hybridization. The plasmid was linearized with *Hind*III, and minus-strand RNA was transcribed with SP6 polymerase (Riboprobe kit; Promega, Madison, Wis.) in the presence of [32 P]UTP (800 Ci/mmol; Amersham, Chicago, Ill.). Ten microliters (2×10^4 cells) was mixed with 2.5 μ l of probe, yielding a final concentration of 4 M guanidine isothiocyanate. Hybridization was performed at 26°C for 10 to 16 h. RNase solution [$2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1 M EDTA, 20 μ g of RNase A (Sigma) per ml, 20 U of RNase T (Sigma) per ml, 50 μ g of poly(A) per ml] was added after hybridization, and the mixture was incubated for 30 min at 37°C. RNA-RNA hybrids were cold precipitated in 30% trichloroacetic acid-6% NaPP_i and collected on Whatman filters. The filters were washed twice with trichloroacetic acid-NaPP_i and once with ethanol and then dried, and radiolabeled RNA was counted. Control samples consisting of uninfected N18 cells were processed and hybridized in an identical manner.

Statistical analysis and surface predictions. Results were analyzed for statistical significance by Student's *t* test with StatView 512 software (Brain Power, Inc., Calabasas, Calif.). Analysis of changes in surface probability and sec-

FIG. 2. Mortality curves after i.c. and s.c. inoculation of 1- to 2-day-old mice with 1,000 PFU of TE or TES. The mean time of death ($\pm$ standard error) after s.c. inoculation was 3.6 ± 0.08 days for TE ($n = 77$) and 4.8 ± 0.22 days for TES ($n = 47$) ($P = 0.0001$). The mean time of death after i.c. inoculation was 2.1 ± 0.06 days for TE ($n = 30$) and 2.6 ± 0.15 days for TES ($n = 30$) ($P = 0.0005$).

ondary structure was performed with MacVector software (IBI, New Haven, Conn.).

RESULTS

Effect of a Gly-to-Arg change at E2 position 172 on mortality in newborn mice and on virus growth in vivo and in vitro. Newborn mice inoculated s.c. live significantly longer after infection with strain TES (mean $\pm$ standard error, 4.8 ± 0.22 days) than after infection with strain TE (3.6 ± 0.08 days) ($P = 0.001$) (18). Survival time is also longer after i.c. infection of newborn mice with TES (2.6 ± 0.15 days) than after i.c. infection with TE (2.1 ± 0.06 days) ($P = 0.0005$) (Fig. 2). These data suggest that the amino acid change from Gly to Arg at position 172 of the E2 glycoprotein leads to a decrease in neurovirulence. Analysis of the growth of both viruses in the brains of newborn mice (Fig. 3) showed the onset of new virus formation within 4 h after infection with TE but not until 8 h after infection with TES. Eventually both viruses grew to similar amounts in brain tissue, and all mice died of encephalitis. Viral antigen was abundant in brain tissue at 48 h postinfection, as shown by immunocytochemical staining, and TE and TES showed similar distributions. Primary replication in muscle tissue did not appear to play a role in the difference in virulence, since growth was similar in muscle and blood after s.c. inoculation (Fig. 3).

To determine whether TE and TES differed in their growth in vitro, each virus was tested in three cell lines: BHK-21,

the cell line used for routine laboratory propagation of Sindbis virus; L-929, a murine fibroblast cell line; and N18, a cloned murine neuroblastoma cell line (Fig. 4). The two viruses showed similar growth patterns in BHK-21 cells and L-929 cells. However, TE grew more rapidly than TES in N18 cells infected at either a low or a high MOI (Fig. 4). As with growth in newborn mouse brain tissue, the initiation of growth of TES was slower than that of TE, but the amounts of virus eventually produced were similar. These data suggest that the Arg at E2 position 172 affects the replication of virus primarily in cells of neuronal rather than nonneuronal origin and that this occurs by delaying virus replication.

Differences in viral RNA synthesis. To begin to determine the point in the replicative cycle of Sindbis virus which is influenced by the amino acid occupying position 172, the time course of the synthesis of viral RNA in N18 neuroblastoma cells was examined (Fig. 5). A minus-strand structural region probe was used, since a minus-sense probe hybridizing to both genomic and subgenomic RNA should provide the most sensitive means for detecting virus RNA accumulation. Viral RNA was first detectable 3 h after infection of neuroblastoma cells with TE but was not detectable until 4 h postinfection with TES. Approximately 7 h after infection, the amounts of RNA of the two strains became comparable. These data suggested that replication of TE and TES in neuroblastoma cells differed at a step at or before the initiation of RNA synthesis.

FIG. 3. Growth of TE and TES in brain tissue, hind foot muscle tissue, and blood. Mice were infected i.c. with 1,000 PFU (brain) or s.c. with 1,000 PFU in both hind feet (muscle and blood). Samples were assayed as 10% homogenates.

FIG. 4. Growth of TE and TES in BHK-21, L-929, and N18 cells. BHK-21 and L-929 cells were infected at an MOI of 1.0, and N18 cells were infected at an MOI of either 0.1 (low) or 10 to 20 (high).

Altered neurovirulence is determined by differences in virus binding to neural cells. Assays of virus binding were done to examine whether differences in attachment to receptors on neural cells could explain differences in the patterns of TE and TES growth in these cells (Fig. 6). Within 30 min after addition of ^{35}S -labeled virus to N18 neuroblastoma cells, TE exhibited increased binding compared with TES, a trend which continued and was statistically significant by 1 h (28 versus 20%; $P = 0.0068$). A plateau for binding was reached approximately 2 h postinfection for both viruses. To determine whether these differences in virus binding were unique to N18 cells, virus binding was also analyzed with the mouse neuroblastoma cell line NB41A3 (TE versus TES, 23 versus 14%; $P = 0.025$), newborn mouse brain cells (60 versus 43%; $P = 0.008$), and BHK-21 cells (66 versus 64%; $P = 0.81$). These data are consistent with the hypothesis that differ-

ences in neurovirulence for these two strains of virus are due to a decreased ability of the virus strain with Arg at E2 position 172 to bind to a virus receptor on neural cells.

DISCUSSION

Sindbis virus causes encephalitis of varying severity in mice and provides an excellent animal model of acute central nervous system disease. By studying the range of virulence in strains of Sindbis virus differing by a limited number of amino acids, information about neurovirulence at a genetic level can be obtained. Although several authors have found that single-amino-acid changes in the E1 or E2 glycoproteins can attenuate both virus replication and virulence (6, 18), the mechanism by which any of these changes alters virulence for mice has not been identified. Our studies have shown that the change from Gly to Arg at position 172 of the E2 glycoprotein increases the survival time of infected mice by decreasing the binding of virus to neural cells without affecting binding to nonneural cells. These findings suggest that a region on the E2 glycoprotein affected by the amino acid occupying position 172 is particularly important for binding to an alphavirus receptor on neurons and may affect penetration as well.

Tropism for specific cell types within the nervous system is a recognized determinant of virulence for reoviruses in which the sigma 1 (hemagglutinin) protein of type 1 targets the virus to ependymal cells, leading to mild disease, while the sigma 1 protein of type 3 targets virus to neurons, leading to fatal encephalitis. However, previous studies of the pathogenesis of Sindbis virus infection have shown that virulent NSV and avirulent AR339 in weanling mice and avirulent HRSP and virulent AR339 in newborn mice affect the same regions of the brain and spinal cord and have

FIG. 5. Time course of the synthesis of Sindbis virus RNA in uninfected N18 cells (control) and in N18 cells infected with TE or TES.

FIG. 6. Time course of the binding of ^{35}S -labeled TE and TES to nonneuronal (BHK-21) and neuronal (N18, NB41A3, and newborn mouse brain) cells at 4°C . Symbol: *, $P \leq 0.05$.

neurons as their primary target cells (12, 26). TE and TES in newborn mice also affect the same regions of the brain. Therefore, the differences in virulence between strains of Sindbis virus are more likely the result of changes in the efficiency of replication in neurons or changes in the effects of infection on neurons.

The two recombinant strains of Sindbis virus examined in this study are both virulent for newborn mice and differ only in the time of death after infection. A more dramatic difference in virulence might be expected in the absence of the His-for-Gln substitution at position 55 of E2, a recognized contributor to increased virulence of NSV for weanling mice

(18). Nevertheless, the effect of the attenuating amino acid, Arg, at position 172 of E2 is clear when TE and TES are directly compared. A similar difference in virulence for newborn mice is also apparent when two other virus strains differing at E2 position 172 are compared: TE2 (Gly; 100% mortality with a mean time of death of 5.2 days postinfection) and TE2S (Arg; 86% mortality with a mean time of death of 8.3 days postinfection) (18). These viruses also differ at position 75 of the E1 glycoprotein, and a contribution from this substitution cannot be excluded. Arg at 172 is derived from the HRSP parent, which has a long history of passage in tissue culture. Wild-type strains of Sindbis virus,

FIG. 7. Probability of surface exposure and predicted secondary structure of the region from residue 150 to 200 of the E2 glycoprotein of TE and TES. CF indicates the Chou-Fasman (4) prediction, and RG indicates the Robson-Garnier (9) prediction. CFrg indicates regions of agreement between the two methods.

such as AR339, have Gly at this position. Arg at E2 position 172 did not affect replication in the nonneural cells tested, and therefore this substitution would provide no selective disadvantage for HRSP during routine passaging in cell culture.

The nature of the neuronal receptor(s) for alphaviruses is unknown. Smith and Tignor (27) studied differential binding of the AR86 and AR339 strains of Sindbis virus to several cell lines including N18 (neuroblastoma), C6 (astrocytoma), and nonneural cells. The receptors on all cells were pronase sensitive, but estimation of the numbers of receptors suggested that N18 cells had 27 times more receptors for the more virulent AR86 strain than for AR339. The nature of this receptor has not been further identified. Alphaviruses have a wide host range and are able to replicate in many types of cells. It is likely that they can use more than one cell surface molecule for entry and infection. In vitro, a wide variety of cell types successfully support virus replication, possibly through use of a variety of receptors of variable efficiency. However, in vivo, where conditions of limiting amounts of virus prevail, only a few of the available cell types are infected (14), and the relative efficiency of virus binding to a functionally important cell such as a neuron may be an important determinant of virus virulence. Glial cells are generally spared from infection, especially in older mice (12), suggesting that neurons have a receptor for Sindbis virus which is not present on other cells in the nervous system. The delay in replication for TES under conditions of high as well as low MOI suggests that the efficiency of penetration may differ as well.

These data also suggest that the amino acid composition of the E2 glycoprotein is important in determining efficient binding to neural cells. By computer analysis the region around E2 position 172 is not predicted to be exposed on the surface when occupied by the nonpolar Gly; rather, it is predicted to be exposed on the surface with the charged Arg in this position (Fig. 7). The conformation of the cell attachment site is not known for alphaviruses but is known from crystallographic analysis for a few viruses (11, 17, 24) and in each case appears to be a "pit" or "canyon" on the surface rather than an exposed site. It is the floor of these canyons and pits that is believed to be the site for virus attachment to the cellular receptor (31). The importance of attachment sites being retained in canyons may have to do with their lack of accessibility to antibody (24). Amino acid 172 is near but not within an identified neutralization site (residues 190 to 220) (6, 29) which is predicted to be exposed on the surface of the E2 glycoprotein. Although spatial relationships on the folded molecule are not easily predicted on the basis of the linear sequence, the current data would fit the general picture of neutralization sites exposed on the virion surface near attachment sites which are deep in the molecule. It is possible that the charged Arg disrupts an attachment site or a portion of such a site used for binding virus to an alphavirus receptor on neurons and helps to define one mechanism for decreased neurovirulence of certain strains of Sindbis virus.

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