Pathogenesis of Encephalitis Induced in Newborn Mice by Virulent and Avirulent Strains of Sindbis Virus

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Strains of Sindbis virus differ in their virulence for mice of different ages; this variation is related in large part to variations in the amino acid compositions of E1 and E2, the surface glycoproteins. The comparative pathogenesis of Sindbis virus strains which are virulent or avirulent for newborn mice has not been previously examined. We have studied the diseases caused by a virulent wild-type strain, AR339, and two less virulent laboratory strains, Toto1101 and HRSP (HR small plaque). After peripheral inoculation of 1,000 PFU, AR339 causes 100% mortality within 5 days (50% lethal dose [LD_{50}] = 3 PFU) while Toto1101 causes 70% mortality (LD_{50} = $10^{2.4}$ PFU) and HRSP causes 50 to 60% mortality (LD_{50} = $10^{5.1}$ PFU) with most deaths occurring 7 to 11 days after infection. However, after intracerebral inoculation of 1,000 PFU, Toto1101 is virulent (100% mortality within 5 days; LD_{50} = 4 PFU) while HRSP is not (75% mortality; LD_{50} = $10^{4.2}$ PFU). After intracerebral inoculation, all three strains initiate new virus formation within 4 h, but HRSP reaches a plateau of 10^6 PFU/g of brain while Toto1101 and AR339 replicate to a level of 10^8 to 10^9 PFU/g of brain within 24 h. Interferon induction parallels virus growth. Mice infected with HRSP develop persistent central nervous system infection (10^6 PFU/g of brain) until the initiation of a virus-specific immune response 7 to 8 days after infection when virus clearance begins. The distribution of virus in the brains of mice was similar, but the virus was more abundant in the case of AR339. HRSP continued to spread until day 9. Clearance from the brain was complete by day 17. We conclude that the decreased virulence of HRSP is due to an intrinsic decreased ability of this strain of Sindbis virus to grow in neural cells of the mouse. We also conclude that CD-1 mice do not respond to the antigens of Sindbis virus until approximately 1 week of age. This lack of response does not lead to tolerance and persistent infection but rather to late virus clearance whenever the immune response is initiated.

Sindbis virus (SV), an alphavirus, is relatively nonpathogenic for its various hosts. In humans, infection is often inapparent but occasionally causes arthritis and a rash (7, 18). In mice, infection predictably causes acute encephalitis, but mortality is age dependent (15, 25). Although arthritis in humans may be prolonged or recurrent, there is no evidence that this is due to persistent infection (20). Most wild-type SV isolates, including the prototype AR339 strain, produce an acute, fatal encephalitis in newborn mice and an acute, but nonfatal, monophasic encephalitis in 4-week-old mice (25, 33, 36). However, laboratory strains of SV derived from wild-type isolates may demonstrate reduced virulence for newborn mice (21). For instance, the frequently studied HR (heat-resistant) strain of SV, derived from AR339 by Burge and Pfefferkorn in 1965 (3), does not cause uniformly fatal disease in newborn mice (17).

Previous studies have shown that the laboratory strains HRSP (HR small plaque) and Toto1101, derived from a genetically engineered full-length cDNA clone of SV (26), are both relatively avirulent for newborn mice after subcutaneous (s.c.) inoculation. Mice often recover from infection, and when infection is fatal, death occurs on average 1 to 2 weeks rather than 3 to 5 days after infection (17, 22). Although relative avirulence for suckling mice has been shown in these studies to be related to amino acid sequence differences in the E1 and E2 surface glycoproteins, there is no information on how these differences influence the pathogenesis of infection in newborn mice. To characterize infection of newborn mice by avirulent strains of SV and to determine whether persistent infection is established in

surviving mice, we have compared the virus replication, interferon production, and immune response induced by infection with the avirulent HRSP and Toto1101 strains of SV with the same factors induced by infection with the virulent AR339 strain.

MATERIALS AND METHODS

Viruses. SV strains AR339 (American Type Culture Collection) (SV1A) (17), HRSP (31), and Toto1101 (26) were grown and assayed by measuring plaque formation in BHK-21 cells. Stock viruses contained 10^7 to 10^8 PFU/ml. These viruses have known amino acid differences in the surface glycoproteins E1 and E2 (17, 22, 31) (Table 1).

Mice. Litters of 1- to 2-day-old CD-1 mice of either sex were used (virus antibody free; Charles River Breeding Laboratories, Inc., Wilmington, Mass.). A small number of experiments were repeated with litters of 1- to 2-day-old BALB/c mice (Charles River Breeding Laboratories, Inc.), with similar results being obtained. Mice were inoculated intracerebrally (i.c.) into the right cerebral hemisphere with 1,000 PFU of virus in 0.03 ml of Hanks balanced salt solution containing 1% fetal bovine serum. Control mice were inoculated with 0.03 ml of diluent. Animals inoculated s.c. received 1,000 PFU of virus in 0.03 ml of diluent divided between both hind feet. Mice were observed daily for 14 to 21 days for mortality. Deaths occurring within 24 h of inoculation were considered to be due to other causes and were not considered in the analysis. Fifty-percent lethal doses calculated according to the method of Reed and Muench (24) were determined by inoculating mice with serial 10-fold dilutions of virus either i.c. or s.c. with 9 to 11 1- to 2-day-old CD-1 mice per dilution.

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TABLE 1. Amino acid differences in the E1 and E2 surface glycoproteins of the SV strains used in this study

Strain	Amino acid at indicated position in glycoprotein:						
	E2				E1		
	3	23	172	209	72	75	237
AR339 (SV1A)	Thr	Glu	Gly	Arg ^a	Val	Asp	Ala
Toto1101	Ile	Glu	Gly	Gly	Ala	Gly	Ser
HRSP	Ile	Val	Arg	Gly	Ala	Asp	Ser

^a Other laboratory strains of AR339 (SIN and SB) have Gly at position 209 (6, 32).

Virus titrations. Brains were removed at various intervals after infection to determine virus content. A 20% homogenate was prepared by freezing and thawing the tissue twice, adding an appropriate volume of Hanks balanced salt solution–1% fetal bovine serum, and repeated aspirations through an 18-gauge needle. The virus was assayed by measuring plaque formation of serial 10-fold dilutions on BHK-21 cells. Values for each time point were based on the geometric means of results from two to seven individual mice.

Interferon assays. Interferon activity in the brain was measured as previously described (10) by a vesicular stomatitis virus challenge of L-929 cells after overnight treatment with serial twofold dilutions of acidified and neutralized brain homogenates. Endpoints were determined by staining the cells with crystal violet.

Antibody measurements. Antibody to SV was measured by enzyme immunoassay essentially as previously described (29). In brief, polystyrene plates were coated with polyethylene glycol-precipitated SV (3 µg per well), blocked with 5% fetal bovine serum, incubated with serially diluted serum and then with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (dilution, 1:200; Dako Corp., Santa Barbara, Calif.), and developed with *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.), and optical density was read at 490 nm. Samples were run in triplicate, and the titer was expressed as the highest dilution of serum with an optical density greater than two standard deviations above that obtained with normal mouse serum.

Pathology. Brains from mice infected with AR339 were taken on day 2 after infection, and brains from mice infected with HRSP were taken on days 2, 5, 7, 9, 11, 15, 17, 19, and 21 after infection. Tissue was fixed in 10% buffered Formalin, embedded in paraffin, sectioned sagittally, and stained with hematoxylin and eosin for evaluation of the inflammatory response. Inflammation was graded on a 0-to-4+ scale as previously described (12). Additional adjacent sections were cut for immunohistochemistry and in situ hybridization studies.

Immunohistochemistry and in situ hybridization. Immunoperoxidase staining for SV antigen was performed as previously described (13) by the avidin-biotin system with rabbit anti-SV antibody as the primary reagent. In situ hybridization was performed essentially as previously described (5, 14) with ³⁵S-labeled anti-sense RNA (specific activity, 9 × 10⁸ dpm/µg) transcribed with SP6 polymerase (Stratagene Inc., La Jolla, Calif.) from a DNA construct encoding the structural genes of SV. Slides were developed 4 days after coating with NTB2 emulsion (Eastman Kodak Co., Rochester, N.Y.), stained with hematoxylin, and examined by bright-field and dark-field microscopy.

Statistics. The significance of differences in mean day of

FIG. 1. Virus-induced mortality. Mortality curves for 1- to 2-day-old mice inoculated either s.c. (●) or i.c. (○) with 1,000 PFU of SV AR339 ($n = 20$ [s.c.] and 9 [i.c.]), Toto1101 ($n = 44$ [s.c.] and 63 [i.c.]), or HRSP ($n = 18$ [s.c.] and 20 [i.c.]).

death was determined by Student's *t* test with Statview 512 software (Brainpower, Inc., Calabasas, Calif.).

RESULTS

Virulence. Virulence was determined first by inoculating 1,000 PFU of each of the three virus strains, AR339, HRSP, and Toto1101, s.c. into newborn mice (Fig. 1). Mortality rates at 21 days for HRSP and Toto1101 after s.c. inoculation were 61 and 70%, respectively, with a mean day of death ($\pm$

TABLE 2. Differences in virulence among SV strains as measured by LD₅₀^a 14 days after i.c. or s.c. inoculation

Strain	LD ₅₀ (log ₁₀ PFU) for mice inoculated:	
	i.c.	s.c.
AR339	<0.1	0.4
Toto1101	0.5	2.4
HRSP	4.2	5.1

^a LD₅₀, 50% Lethal dose.

standard error of the mean) of 10.5 ± 1.3 and 7.6 ± 0.7 ($P = 0.048$), respectively, compared with 100% mortality and a mean day of death of 4.5 ± 0.2 for AR339 ($P < 0.0001$ for HRSP; $P < 0.001$ for Toto1101). To determine whether HRSP and Toto1101 would also be avirulent for newborn mice if the brain were infected primarily rather than secondarily, mice were also inoculated with each virus i.c. (Fig. 1). Toto1101 was only slightly less virulent than AR339 by this route, with 100% mortality and mean days of death of 4.3 ± 0.2 and 3.7 ± 0.2 , respectively ($P = 0.03$). In contrast, HRSP remained comparatively avirulent after i.c. inoculation, with only 75% mortality after 14 days and a mean day of death of 7.8 ± 1.3 ($P = 0.02$ for Toto1101; $P < 0.001$ for AR339). These relative differences were confirmed by determining the i.c. and s.c. 50% lethal doses for each virus strain (Table 2). Subsequent studies were performed by i.c. inoculation.

Virus growth. The replication of virus in the brains of mice after i.c. infection was measured (Fig. 2). All three strains initiated new virus formation within 4 h of infection. Growth rates were similar up to 9 h, although Toto1101 had the highest yield at each point during this time. At 9 h after infection, amounts of Toto1101 and AR339 continued to increase to 10^8 to 10^9 PFU/g of brain, while amounts of HRSP plateaued at approximately 10^6 PFU/g of brain. Except for a transient increase to 10^7 PFU/g 36 h after infection, levels of HRSP of about 10^6 PFU/g were maintained until 8 days after infection, at which time virus clearance began (Fig. 3). The virus was not detectable by routine assay of brain homogenates after day 10. Many, though not all, of the HRSP-induced deaths occurred during the first 7 to 8 days after infection when virus replication was ongoing (Fig. 1).

Interferon induction. To determine whether early induction of interferon might account for the attenuation of HRSP replication, brain homogenates were assayed for interferon activity (Fig. 4). Interferon was first detected at 12 h in brains from mice infected with Toto1101, 16 h for AR339, and 20 h for HRSP. In general, interferon levels paralleled virus titers and at 24 h were high for all virus strains, suggesting that interferon was not the primary factor limiting the initial phase of virus replication in HRSP-infected mice. However, interferon was maintained at a relatively high level (dilutions of 1:64 to 1:256) in the brains of HRSP-infected mice through day 9 and may have had a moderating effect on later virus replication and spread within the central nervous system.

Immune response. To determine whether newborn mice infected with HRSP developed an immune response to virus infection and whether this correlated with eventual virus clearance, SV-specific antibody was measured by enzyme immunoassay and cellular immunity was evaluated by the appearance of mononuclear inflammatory cells in the brain (19) (Fig. 3). Both antibody to SV and inflammation were first detected 7 days after infection, coincident with the beginning of virus clearance from the brain. The time course of the immune response was similar after s.c. inoculation of

FIG. 2. Brain virus growth. Amounts of virus in the brains of mice inoculated i.c. at 1 to 2 days of age with 1,000 PFU of AR339 (●), Toto1101 (△), or HRSP (○). Each point represents the geometric mean of the individual titrations of the brains of two to seven mice. Bars indicate the standard error of the mean for each point.

HRSP, with antibody first detectable 7 to 8 days after infection. Mice infected either i.c. or s.c. with AR339 died before detectable antibody appeared in serum.

Virus localization. Differences in virulence might be explained by differences in tropism of HRSP and AR339 for cells of the central nervous system. To assess this possibility, brains were stained by immunoperoxidase to localize viral antigen (data not shown) and by in situ hybridization to localize viral nucleic acid (Fig. 5). No viral antigen or RNA was detected in the brains of uninfected animals. On day 2, viral antigen and RNA were abundant in HRSP-infected mice. Foci of infected cells were found in the cerebral cortex, cerebellum, hippocampus, thalamus, and brain stem as well as nearby muscle. The distribution of viral antigen and RNA was similar in mice infected with AR339, which had more abundant virus in each of the infected areas (Fig. 5A). On days 5 and 7 after HRSP infection, the virus was widely distributed in the thalamus, brain stem, colliculus, and cortex (Fig. 5B). By day 9, viral antigen was difficult to locate, but viral RNA could still be readily identified by in situ hybridization through day 15 (Fig. 5C and D). A search for evidence of persistent infection showed no evidence of virus beyond day 15, suggesting that clearance was complete.

DISCUSSION

These studies have shown that HRSP and Toto1101, laboratory strains of SV, are less virulent for newborn mice after s.c. inoculation than wild-type strains of SV and that HRSP is also less virulent after i.c. inoculation. The decreased virulence of HRSP is associated with lower levels of

FIG. 3. Immune response and virus clearance after HRSP infection. Mice were inoculated i.c. at 1 to 2 days of age with 1,000 PFU of HRSP. The amounts of virus in the brain, antibody in serum as measured by enzyme immunoassay (EIA), and inflammation in the brain (scored as - to +++) were determined. Each point represents the mean of three independent determinations (virus and inflammation) or multiple assays of serum pooled from three mice (antibody). Inflammation never reached the levels (+++++) present in weanling mice infected with AR339 (12, 19).

virus replication in the brain, which is due not to an earlier induction of interferon or specific antiviral immune response but rather to an intrinsic difference in virus growth in neural cells. Furthermore, infection of neonatal animals does not lead to long-term persistence of the virus, even in the face of

a slow immune response to viral antigen. These studies suggest a fundamental difference in the ability of the HRSP strain of SV to replicate in neural tissue rather than a difference in induction of host responses.

Rapid induction of interferon has been suggested as a mechanism of decreased virulence on the basis of comparisons of plaque variants of SV (23). In one study, a large-plaque strain of AR339 was less virulent and induced interferon more rapidly than a small-plaque strain did (23). However, we found that interferon was slower to appear in the brains of mice infected i.c. with avirulent HRSP than with virulent Toto1101 or AR339. In general, as previously reported for other SV systems (11, 25, 35), the amount of interferon in the brain correlated with the amount of virus present. Interferon may, however, account for the failure of HRSP to continue to increase in amount in the brains of infected mice after the first 36 h of infection. High levels of interferon are present by 24 h and are maintained throughout the 8 to 9 days of active infection until the specific immune response appears.

The delayed clearance of HRSP from brains of newborn mice is very similar to the delayed clearance of AR339 from the brains of 4-week-old mice immunosuppressed with cyclophosphamide (19). AR339 is not virulent for 4-week-old mice (11, 15) and is normally completely cleared in 6 to 7 days, with the onset of the antiviral immune response occurring 3 to 4 days after infection. Cyclophosphamide immunosuppression delays appearance of the immune response until 7 to 8 days after infection and delays viral clearance until 11 to 12 days after infection, with persistent viral titers of 10^6 PFU/g of brain until virus clearance begins (19). In both situations, the amount of virus in the brain never reaches that of the virulent strain (AR339 for newborn or NSV for 4-week-old mice) despite the absence of an immune response. This suggests intrinsic virus-encoded differences in replicative potential, either in the number of cells infected initially, in the amount of virus produced by each infected cell, or in the ability of the virus to spread

FIG. 4. Brain interferon. Amounts of interferon activity in the brains of mice inoculated i.c. at 1 to 2 days of age with AR339, Toto1101, or HRSP. Each point represents the geometric mean of values obtained for the brains of three individually assayed mice. Amount of interferon is expressed as the highest dilution of a 10% brain homogenate which protected L cells from a vesicular stomatitis virus challenge.

FIG. 5. In situ hybridization of SV RNA in sagittally sectioned brains of newborn mice inoculated i.c. at 1 to 2 days of age with 1,000 PFU of AR339 (A [section obtained on day 2 shown]) or HRSP (B [day 7], C [day 9], and D [day 15]). The locations of the brain stem (bs), thalamus (t), and cerebral cortex (c) are indicated. For AR339 (A), the virus is also present in meninges (m) and muscles of the head (h). Dark-field illumination; magnification, ca. $\times 14$.

rapidly to additional neural cells before induction of an amount of local interferon sufficient to inhibit further spread.

The entire sequence of HRSP and the sequences of the structural regions of AR339 and Toto1101 are known. Amino acid sequence differences exist at four positions in the surface glycoprotein E2 (positions 3, 23, 172, and 209) and at three positions in the surface glycoprotein E1 (positions 72, 75, and 237) (Table 1) (17, 22, 31). Nonstructural-region changes have not yet been identified. Toto1101 is relatively avirulent, like HRSP, after s.c. inoculation but, unlike HRSP, is virulent after i.c. inoculation. This suggests that Toto1101 has attenuating properties which affect replication at peripheral sites or invasion of the central nervous system from the periphery. Amino acid changes in both E1 and E2 have been shown to affect the virulence of these viruses for newborn mice (17, 22). Few of the changes have been independently evaluated for their specific contributions to virulence.

In E2, Arg substituted for Gly at position 172 decreases binding to neuronal cells (P. C. Tucker and D. E. Griffin, unpublished observations) and may contribute to the avirulence of HRSP after i.c. inoculation. In E1, the amino acid at position 72 is an important determinant of the pH of fusion of the viral membrane with the cell membrane, with Ala requiring a lower pH than Val at this position (2), and could potentially alter the efficiency of entry of Toto1101 and HRSP into cells. The change from Ala to Ser at position 237 in E1 appears to be important for age-dependent virulence after s.c. inoculation (17), but the mechanism has not been identified. Important differences in the nonstructural region are documented for virulence in weanling mice (17) and cannot be ruled out as important for newborn mice as well. Further detailed analysis of the molecular basis for these

differences in virulence will depend on further construction of recombinant viruses differing at only a single position.

Newborn mice do not mount an immune response to SV until approximately 7 days of age, suggesting that the immunologic repertoire of T or B lymphocytes does not include the relevant variable-region rearrangements until that age. Previous studies of the role of the immune response in the age-dependent mortality of AR339 infection in mice have shown that fully susceptible 1-week-old mice develop virus-specific antibody and cellular immunity 3 to 4 days after infection, as rapidly as resistant older mice (9). The relatively low level of HRSP replication does not account for the late antibody development, since AR339 replicates to similar levels (10^6 to 10^7 PFU/g of brain) in 4-week-old mice which develop antibody by 3 to 4 days after infection (9, 25). Many other antigens also are not in the murine repertoire at birth but are acquired in a patterned and reproducible fashion during the first weeks of life (8, 16, 27, 28, 30). Most extensive studies with viral antigens have used influenza virus hemagglutinin and show that the antibody repertoire at 12 to 14 days of age is only 10% of the adult repertoire (4). Since we assayed antibody by enzyme immunoassay, an antibody response to any virion antigen should have been detected.

The outcome of infection of an immunologically immature animal with an avirulent strain of SV is entirely different from the outcome of infection of neonatal mice with the noncytopathic lymphocytic choriomeningitis virus. In the latter instance, the virus is never cleared and persistent infection of the central nervous system and other organs is established (34). This may be due to selection of a strain of virus which suppresses the eventual appearance of cellular immune responses necessary for virus clearance (1), an

event which does not occur during prolonged infection with SV (1), or to some other intrinsic difference between these infections. These studies show that persistent infection is not the inevitable outcome of viral infection of a neonatal animal with an avirulent virus.

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