An Avirulent G1 Glycoprotein Variant of La Crosse Bunyavirus with Defective Fusion Function

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La Crosse virus, a member of the California serogroup of the family *Bunyaviridae*, causes encephalitis in humans and laboratory rodents. A variant virus (V22) selected with a monoclonal antibody against the large (G1) glycoprotein showed diminished neuroinvasiveness after peripheral inoculation. This variant has an alteration in its fusion function, requiring a lower pH for the activation of fusion and demonstrating reduced efficiency of cell-to-cell fusion of BHK-21 cultures. V22 was studied in detail following the infection by intraperitoneal or intracerebral routes in suckling, weanling, or adult CD-1 mice. It exhibited a marked reduction in its ability to replicate in striated muscle and to produce viremia; however, after intracerebral injection V22 virus replicated almost as rapidly in brain as its parent, La Crosse virus. V22 virus thus represents an example of reduced neuroinvasiveness associated with an alteration at a specific epitope of the G1 glycoprotein. This same epitope also influences the fusion activity of the glycoprotein.

The study of virus variants can provide a powerful approach to the delineation of the molecular basis of virus virulence. The ease of selection of antigenic variants has allowed investigation of the effect of alterations of virus functions on the tropism and virulence of different virus families. Several studies have shown that variants selected with monoclonal antibodies have reduced virulence. In the rabies system (2, 32), the genome of the variants with decreased pathogenicity has been sequenced (4), and as with other monoclonal variants (3), the alteration is a single nonredundant base substitution leading to a single amino acid change. The biological function that is inhibited has not yet been studied. Several reovirus variants (30) selected with a single monoclonal antibody show a dramatic decrease in virulence when injected intracerebrally (i.e.) in suckling mice, and it has been postulated that this decrease in pathogenicity is due to altered receptor binding (29). We selected a group of monoclonal antibody variants of La Crosse (LAC) virus (10), and among them a variant selected by one monoclonal antibody (807-22) showed decreased neuroinvasiveness, that is, decreased ability to cause a central nervous system (CNS) infection after peripheral inoculation. Prototype variant 22 (V22) virus was also inefficient at mediating cell-to-cell fusion either from within (FFWI) or without (FFWO). This study presents a comparison of V22 and parent LAC viruses with respect to fusion activity and pathogenesis in mice.

MATERIALS AND METHODS

Monoclonal antibodies. The procedures used for the generation of monoclonal antibodies against LAC virus have been described elsewhere (10). Briefly, weanling BALB/c mice were infected with approximately one 50% lethal dose (LD₅₀) of LAC virus (i.e., LAC original, passage 6), and the animals surviving 3 weeks later were rechallenged with LAC virus. The spleen cells were fused with mouse myeloma cells (17, 19) 2 to 4 days later, and antibody-secreting colonies were selected by enzyme-linked immunosorbent assay (ELISA) and then cloned in agarose. The protein specificity

of the monoclonal antibodies was determined by immunoprecipitation of solubilized, [³H]leucine-labeled, LAC virus-infected cell extract. All of the antibodies were directed against either the large (G1) glycoprotein or the nucleocapsid (N) protein (10, 11, 18, 25).

Selection of variant 22 (V22) virus. LAC original virus has been described previously (15). Tenfold dilutions of LAC virus, in the form of a 10% suspension of suckling mouse brain, were combined with a low dilution (1:5 or 1:10) of 807-22 monoclonal ascitic fluid and held for 30 min at room temperature. The virus-antibody mixture was inoculated onto a monolayer of BHK-21 cells on six-well plates and rocked gently for 60 min at room temperature. The inoculum was then removed, and the monolayer was overlaid with Eagle modified minimal essential medium (MEM) containing 2% fetal calf serum (FCS) and a final concentration of 0.5% agarose (Seakem HE; FMC Corp., Marine Colloids Div., Rockland, Maine). Isolated plaques appearing at the end point of neutralization were picked and purified twice, using antibody during the initial stage of infection of each purification. The frequency of selection was calculated as (titer of LAC stock + ascitic fluid)/titer of LAC stock.

Variants were selected with monoclonal 807-22 on two occasions 1 year apart. Unless specified otherwise, all of the studies described here were done with a single clone designated V22. A second clone (V22F), selected independently, was used to confirm that these results apply to other variant viruses selected with this antibody and are not characteristic of a single spontaneous mutation. V22F was selected from a tissue culture stock of LAC, whereas V22 was selected from a 10% suspension of LAC-infected suckling mouse brain homogenate.

Mice. Outbred albino Swiss mice (CD-1; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) of various ages were used. All animal studies were done with single plaque-purified tissue culture stocks of LAC or V22. LD₅₀ titers were calculated by inoculating five weanling or adult mice or one litter of suckling mice with 10-fold dilutions of LAC or V22 viruses. For pathogenesis studies, suckling mice (48 to 72 h old) were inoculated intraperitoneally (i.p.) with 2,200 PFU of either virus; weanling mice (3 weeks old)

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were inoculated i.p. with 10^5 PFU, and adult mice (8 to 10 weeks old) were inoculated i.c. with 1,000 PFU of either virus. Mice were infected with calibrated inocula of 0.05 or 0.02 ml.

Survival. Animals of each age group were infected, and the survivors were counted every 8 h for 15 days. By using 25 to 40 mice per group, mean survival times and standard deviations were computed.

Tissue titrations. The details of tissue titrations have been described previously (15). Mice were infected, sacrificed by decapitation, and bled into a tube containing 300 U of heparin. Selected organs (brain, quadriceps muscle, liver, and heart) were dissected from three mice and prepared as 10% suspensions in MEM with 2% FCS. Samples were assayed by determining the number of plaques on BHK-21 cells, and the titers were recorded as PFU per milligram of tissue or microliter of plasma; the assay had a sensitivity of 1 PFU per mg or μ l.

Plasma antibody response. The antibody response to inoculated virus was determined in the weanling mice used for tissue titration. Serial twofold dilutions of plasma were tested by ELISA with band-purified virus as the antigen as described previously (8a). Titers were recorded as the highest dilution of plasma which gave an optical density reading of at least twice the background reading of preinfection plasma samples.

Immunofluorescent assay techniques. Immunofluorescent assay methods for observing viral antigen in frozen sections of infected mouse tissue have been described previously (15). The indirect immunofluorescent method was used with hyperimmune rabbit anti-LAC antiserum (neutralization titer, 1:3,200) in the first step and a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (light and heavy chain specific; Cappel Laboratories, Cochranville, Pa.) in the second step. Both reagents were used at a dilution of about 1:16, and Evans Blue (final concentration, 0.5% [vol/vol]) was added to the conjugate as a counterstain. Brains were cut in parasagittal sections, and sections of the torsos of suckling mice were cut in the transverse plane. Infection of skeletal muscle in suckling mice was evaluated by counting positive cells in three animals at each time point and determining an average number of infected cells. Controls for specificity were regularly included; the only non-specific staining seen was confined to chondrocytes, cartilage, skin, and intestinal mucosa.

Histology. Moribund mice were perfused, and the tissues were stored in Bouin's solution, embedded in paraffin, and stained with Luxol fast blue and hematoxylin and eosin (15).

Plaque titers of viruses. The procedures used in determining plaque titers of LAC, V22, and V22F are described in detail elsewhere (15).

Cell-to-cell FFWO. The quantitation of FFWO with LAC virus has been described previously (9).

FFWI. A subconfluent monolayer of BHK-21 (clone 13) cells on 96-well Costar plates (2.2×10^4 cells per well) was inoculated with LAC or V22 at a multiplicity of infection (MOI) of 1.0, kept at room temperature for 1 h, washed with phosphate-buffered saline (PBS), and then incubated with MEM plus 2% FCS at 35°C. At 16 h after infection the cells were washed with PBS, exposed to prewarmed (37°C) MEM with 10 mM morpholinoethanesulfonic acid adjusted to the appropriate pH with NaOH, and incubated with MEM plus 2% FCS at 37°C for 30 min. The cells were then fixed and stained with Giemsa (Difco Laboratories, Detroit, Mich.). The nuclei and cells were then counted, and the fusion index (FI) was determined (31) as $FI = 1 - (C/N)$, where C and N

are the numbers of cells and nuclei, respectively. Approximately 1,700 nuclei were counted for each data point (see Fig. 2 and 3).

RESULTS

Selection and characterization of V22 virus. Antigenic variants were selected with antibody 807-22 at a frequency of $10^{-5.8}$, which was slightly higher than the average frequency for all LAC-neutralizing monoclonal antibodies available in our laboratory (10). All of the antigenic variants obtained with our panel of monoclonal antibodies were screened for any alteration in virulence by inoculating 1,000 PFU subcutaneously into suckling mice. The parent LAC virus and all other variants killed 100% of the inoculated mice at this dosage. V22, which killed about 20% of the animals, was then formally tested by determining the PFU/LD₅₀ ratio for suckling, weanling, and adult CD-1 mice after i.p. and i.c. inoculations (Table 1). When compared with LAC virus in parallel experiments, there was a 100-fold to 1,000-fold reduction in mortality after the peripheral inoculation of V22 virus. The LD₅₀ after intracranial inoculation of V22 was not diminished consistently, indicating that the salient biological defect is reduced ability to penetrate the CNS. Despite the low mortality after peripheral injection of V22, adults and weanling mice acquired an immunizing infection, since they survived a potentially lethal challenge with LAC given about 3 weeks after the initial infection with V22 and they developed antiviral antibody as measured by ELISA.

V22 grew in BHK-21 cells at a rate equivalent to that of the parent virus, but peak titers were usually 0.5 log₁₀ lower than those for LAC, regardless of the initial MOI (Fig. 1). When growth under agar was compared, the V22 plaques were noticeably smaller (Fig. 1).

Since these data suggested that the deficiency responsible for the decreased virulence was not poor interaction with cellular receptors, we looked at another major glycoprotein function. Cell-to-cell fusion has been used extensively for the demonstration of fusion activity in several virus families (1, 21, 31). We recently demonstrated (9) that LAC virus is capable of fusing cells (FFWO) when absorbed at 4°C and exposed to acidic pH. FFWI, that is fusion of cells that have been actively infected, also occurs with LAC virus, and the pH characteristics are similar to those of FFWO. In our assay, fusion of cells with LAC begins when the pH is dropped to 6.3 and is maximal, in the 30 min allotted, when the pH is below 6.0. V22 virus was able to mediate FFWI, but the pH of optimal fusion was lower, with fusion of cells noted only after the pH was dropped to 5.8; maximal fusion occurred at pH 5.0. The fusion efficiency of V22, even at this

TABLE 1. Virulence of LAC and V22 viruses in CD-1 mice^a

Route of injection	Virus	Virulence (log ₁₀ PFU/LD ₅₀) in mice at age:		
		Suckling	Weanling	Adult
Intracerebral	LAC	-0.3	0.3	0.52
	V22	0.9	1.77	1.97
Intraperitoneal	LAC	0.49	3.71	3.90
	V22	2.35	>6	>7

^a A tissue culture stock of each virus with a titer of 2×10^8 PFU/ml was used for all experiments. Swiss albino mice (CD-1) were inoculated with the appropriate volume (i.e., 0.02 ml; i.p., 0.05 ml) of virus diluted in 0.75% bovine albumin-PBS. Five weanling or adult mice were inoculated per 10-fold dilution. Suckling mice were tested in groups of one litter per dilution.

pH, was lower than the fusion efficiency of LAC (Fig. 2). FFWI at pH 3.8, 4.0, and 4.5 was also less efficient for V22, suggesting that the deficiency was not just due to an alteration of the optimal pH for fusion but also to reduced efficiency.

Because V22 grows to a lower titer than LAC in BHK-21 cells, we compared in other experiments the maximum fusion obtained with each virus at the pH of peak fusing activity regardless of the time of infection. For LAC, max

FIG. 1. Comparative growth of LAC and V22 viruses in BHK cells. (Top) LAC or V22 virus was inoculated for 1 h on BHK-21 cells at an MOI of 1.0 PFU per cell and maintained at 35°C in MEM supplemented with 2% FCS. At the designated intervals, supernatant fluids were collected and frozen at -80°C until assay (see below). Symbols: ●, LAC; ▲, V22. (Bottom) Plaques of LAC and V22 viruses on BHK-21 cells grown on six-well plates by inoculating for 1 h at room temperature and then overlaying after removal of the inoculum, with medium (MEM plus 2% FCS) containing 0.5% agarose (Seakem HE; Marine Colloids). On day 3 after infection, the cells were stained with 0.1% neutral red. For other experiments not designed to compare plaque size, LAC plaques were read on day 2 and V22 plaques on day 3 after infection.

FIG. 2. FFWI by LAC and V22 viruses. A subconfluent monolayer of BHK-21 cells on 96-well plates (2.2×10^4 cells per well) was inoculated with LAC or V22 at an MOI of 1.0, kept at room temperature for 1 h, washed with PBS, and then incubated with MEM plus 2% FCS at 35°C. At 16 h after infection, the cells were washed with PBS, exposed to MEM at the appropriate pH, and incubated for 30 min. The cells were then fixed and stained, and the fusion index was determined (see the text) after counting the nuclei and cells (ca. 1,700 nuclei per data point). The 95% confidence limit for all points was ± 0.05 . Symbols: ●, LAC; ▲, V22; ○, uninfected control.

imum fusion was obtained at 16 h after infection at an MOI of 1.0 PFU per cell. The fusion index, an index of the extent of cell fusion that takes into account both the number of cells fused and the size of the average polykaryon (31), was 0.95 (1.0 being the theoretical maximum, with an infinite number of nuclei in one giant polykaryon). For V22 virus, peak fusion was obtained at 17 h, just before the cells begin to show extensive cytopathic effect, and the fusion index was 0.76. The pH requirements for FFWO were identical to those for FFWI for both LAC and V22.

A comparison of the amounts of virus protein required for FFWO was then undertaken. The results (Fig. 3) showed that greater amounts (fourfold greater at an index of 0.5) of V22 are required for FFWO and that the maximum level of fusion with the variant was lower than with LAC (0.65 versus 0.84).

To determine whether the V22 epitope was consistently associated with altered biological properties, an independently selected variant, designated V22F, was tested in mice. V22F resembled V22 virus in its reduced virulence (data not shown). The pH requirements for FFWI with V22F virus were identical to those of V22 virus, and V22F also formed small plaques under agarose.

Pathogenesis of V22 virus. Since viral replication and susceptibility to infection by bunyaviruses are age related

FIG. 3. Comparison of FFWO by LAC and V22 viruses. Virus was grown in BHK-21 cells and precipitated from clarified tissue culture supernatant with 7% polyethylene glycol 8000 in 362 mM NaCl, banded in a sucrose gradient (20 to 70%) in 1 M NaCl-10 mM Tris (pH 7.4). Concentrations of purified virus were measured after disruption in 0.1% Triton X-100 by the Bio-Rad method with bovine serum albumin as a standard. The virus was absorbed in a volume of 20 µl per Teraskaki well for 1 h at 4°C, and the cells were exposed to either pH 5.5 (LAC) or 5.0 (V22) buffer, neutralized, and incubated at 37°C for 30 min. The 95% confidence limit for all points was ± 0.05 . Symbols: ●, LAC; ▲, V22.

(16), we sought to demonstrate the differences between LAC and V22 viruses by inoculating various age groups of mice by different routes.

To study the differences in replication in peripheral tissues under conditions of uniform fatality, we inoculated suckling mice, a very permissive age group, with 2,200 PFU i.p. To accentuate differences in survival, 3-week-old weanling mice were inoculated i.p. with 10⁵ PFU. To investigate replication restricted to the CNS, we inoculated adult mice (8 to 10 weeks old) with 1,000 PFU i.c., since virus cannot be

FIG. 4. Survival of CD-1 mice inoculated with LAC or V22 viruses. Each curve was based on a group of 25 to 40 mice.

isolated from nonneural tissues in this age group. We then contrasted the two viruses by comparing survival time, tissue titration, immunofluorescence, serological response, and histological data.

Survival. Suckling mice inoculated i.p. with 2,200 PFU of either virus died rapidly, with a median survival time of 3.3 ± 0.3 days (Fig. 4). The majority (85%) of weanling mice inoculated i.p. with 100,000 PFU of V22 survived infection, compared to a minority (35%) of the mice inoculated with LAC virus. Sick animals sat hunched, moved slowly, circled repetitively, were paretic, or had generalized seizures. Moribund animals exhibited irregular breathing, paralysis, and unresponsiveness. Adult mice inoculated with LAC virus died slightly more rapidly than mice inoculated with V22 virus (mean survival times of 5.0 ± 0.3 and 6.0 ± 0.3 days, respectively).

Tissue titrations. To follow the course of infection, mice were sacrificed at regular intervals, and selected tissues were titrated for virus content. The data are presented in Fig. 5 through 7; the timepoints for asymptomatic mice represent pools of three animals, whereas sick or moribund groups represent pools of one, two, or three animals.

Replication in fatally infected suckling mice inoculated with 2,200 PFU is shown in Fig. 5. Of particular note is the

FIG. 5. Replication of LAC and V22 viruses in suckling CD-1 mice after i.p. injection of 2,200 PFU. Each point represents a pool of tissues from three mice.

replication in peripheral sites. LAC virus was isolated in high titers from all of the extraneural tissues examined. However, V22 virus was only detected in peripheral tissues known to support LAC replication (i.e., skeletal muscle and heart) and then only in moribund animals. We were unable to isolate V22 from either plasma or liver at any time. Virus could be detected in the brains of animals inoculated with either strain within 24 h after infection, and titers subsequently rose to high levels, although V22 lagged slightly behind LAC virus. Replication in the brain probably reflected the seeding of the CNS by either virus during the initial passive viremia immediately after inoculation, thus eliminating the need for peripheral replication and explaining the similarity in growth curves in the CNS.

The replication of virus in weanling mice inoculated i.p. with 100,000 PFU is summarized in Fig. 6. Because the outcome varied, mice were sorted into three groups at the time of sacrifice: asymptomatic, sick, and moribund. Only the brain titers are plotted since, with the exception of traces of LAC virus from quadriceps tissue, we were unable to grow either virus from plasma or skeletal muscle. When the two viruses were compared, LAC was detected in the brain at 2 days after infection, but V22 was not isolated until 4 days after infection. Subsequently, LAC grew to higher titers in brain than did V22 virus. Among mice inoculated with each virus, there was a hierarchy of titers, moribund mice demonstrating the highest and asymptomatic mice the lowest levels.

To study differences in replication within the CNS, adult animals were inoculated i.c. with 1,000 PFU (Fig. 7). The course of infection for both viruses was similar, but LAC virus grew slightly better, with a peak brain titer of $10^{5.0}$ PFU/mg compared with $10^{4.5}$ PFU/mg for V22 virus. Thus, V22 virus had only a slightly diminished ability to replicate in the CNS.

To determine whether V22 virus underwent phenotypic reversion during mouse passage, one isolate (Fig. 6; V22, day 5, moribund) was assayed in the presence and absence of monoclonal antibody 807-22; the titers were identical. In addition, all V22 isolates continued to demonstrate the small-plaque characteristic of V22 virus.

Immunofluorescent assay observations. The accumulation of viral antigen in peripheral and central tissues was determined by indirect immunofluorescence to corroborate the tissue titration data and to examine the CNS for differences

FIG. 6. Replication of LAC and V22 viruses in weanling CD-1 mice injected i.p. with 100,000 PFU. Titers are shown for brain only since replication was negligible in extraneural tissues (see the text).

FIG. 7. Replication of LAC and V22 viruses in adult CD-1 mice after i.c. injection of 1,000 PFU.

in the localization of infection. The following summary is based on a detailed tabulation of the results. In suckling mice, cross-sections of head, thorax, and abdomen were stained to document the decreased presence of V22 in skeletal muscle. Although V22 virus was able to replicate in skeletal muscle, V22 antigen-positive cells occurred less frequently and they were localized to foci in the paraspinal muscles in comparison to LAC virus, which was widely distributed in muscle. An actual count of antigen-positive skeletal muscle cells performed 2 days after infection on three mice per virus gave a mean of 345 for LAC virus-infected animals and a mean of 48 for V22 virus-infected animals (data not shown). LAC antigen was also occasionally detected in heart muscle. In addition, the parasympathetic plexus of the gut and the periaortic ganglia were consistently positive in animals infected with either virus. Skeletal muscle in weanling mice was consistently negative for antigen. No other peripheral tissues were examined.

The infected CNS of mice of each age group demonstrated a very similar pattern regardless of the virus strain or the route of inoculation. In suckling mice, antigen first appeared at 48 h in the striatum and rhinencephalon, and by days 3 and 4 it was widespread throughout the neuraxis. All of the cells seemed to stain in the moribund suckling animals, but there was sparing of the major white-matter tracts. Weanling and adult mice demonstrated a more limited involvement of the brain, such that individual cells could be recognized. There were virtually no differences between moribund V22 and LAC virus-infected suckling or weanling animals. Adult mice demonstrated more LAC than V22 antigen, a finding that is consistent with the slightly higher titers reached by the parental strain (Fig. 7). V22 virus did not show altered tropism within the CNS; like LAC virus, it infected neuronal cells and spared the white matter.

Plasma antibody titers. The plasma antibody responses of weanling mice were determined by ELISA. Infection initiated a brisk antibody response that was first evident at 4 days and rose to high levels by 13 days after infection (Fig. 8). The responses to both viruses were very similar.

Histological observations. Pathological examination was limited to the brains of moribund mice, since LAC virus does not cause extraneural lesions and premoribund mice show few and variable CNS alterations.

Age has a major influence upon the brain lesions produced by LAC virus, as described previously (16). Suckling mice

FIG. 8. ELISA titers of plasma antibody against LAC virus in weanling CD-1 mice infected i.p. with 2,200 PFU of LAC or V22 viruses. Each point represents a pool of sera from three mice. Titers are expressed as the highest dilution which gave an optical density reading at least twice the background level (see the text).

exhibited an overwhelming neuronal necrosis often accompanied by hemorrhages; the necrosis was seen predominantly in the cerebellum, cerebral cortex, and olfactory bulb and occasionally in the hippocampus or thalamus. There was little concomitant inflammation.

Weanling and adult mice had similar lesions of two distinct types. Focal necrotic lesions of variable severity, accompanied by inflammation, were seen in the hippocampus and olfactory bulb. About one-half of the animals also showed striking, sharply demarcated focal areas of status spongiosus in the midbrain or pons and occasionally in the thalamus and rhinencephalon.

Mice that were moribund after infection with V22 virus showed lesions very similar to those described above for parent LAC virus (Table 2). There were more lesions in the mice infected with LAC virus (52% had moderate or severe lesions) than in those infected with V22 virus (22% had moderate or severe lesions).

DISCUSSION

Although the exact role of fusion in the biology of enveloped viruses is not well understood, there is general agreement that fusion is intimately involved with the early events in viral penetration (6, 12, 14, 20, 22, 23). The envelope glycoproteins have been shown to mediate this function in several well-characterized systems (1), and hydrophobic sequences have been identified as essential peptides for fusion (8, 26). In both the *Orthomyxoviridae* and the *Paramyxoviridae* a conformational change in the fusion protein is necessary for the activation of fusion (13, 28).

The information currently available about the LAC virus glycoproteins is insufficient to predict which of the two glycoproteins contains the putative hydrophobic peptide that mediates this function. The complete sequence of the middle-sized RNA segment, which codes for the glycoproteins (7, 27) of the closely related Snowshoe Hare virus, shows that this RNA could code for a hydrophobic sequence at the carboxy terminus of one glycoprotein, the amino terminus of another, or within either of the two proteins (5). By using amino acid-specific proteases that digest G1 while still attached to the viral membrane, we have shown that the

exposure of virions to an acid environment leads to different cleavage patterns. Similarly, four of the G1 monoclonal antibodies previously generated, including 807-22, show diminished binding (as determined by ELISA) after the acidification of LAC virus, and the pH of change correlates with the pH of activation of the fusion function (8a). We interpret these findings as showing that the large glycoprotein G1 undergoes a conformational change at this pH. The identity of the actual fusion protein remains to be determined, since the conformational change elicited by acid may expose the small glycoprotein G2 or it may expose other sites on the G1 itself. In any event, the altered fusion pH in a G1 monoclonal variant confirms that the large protein is intimately involved with this function.

We demonstrated that a monoclonal variant deficient in the fusion function shows altered pathogenicity in mice. We have previously shown (15) that the injection of mice with LAC virus by a peripheral (i.p. or subcutaneous) route causes an infection characterized by plasma viremia secondary to replication in muscle. V22 virus was unable to replicate efficiently in muscle, and it did not establish viremia. Nevertheless, once the virus reached the CNS, it replicated with only slightly diminished efficiency in comparison with the parental strain. The distribution of antigen in the brain was similar to that of parent LAC virus, and the pathological changes, though less severe than with the parent strain, were typical of an acute viral encephalitis. The antibody response was similar in both viruses. Thus, we have established that V22 has diminished neuroinvasiveness with only minimal loss of neurovirulence. In this respect it resembles an attenuated strain of the related Tahyna virus, which also shows decreased replication in muscle.

Since the exact role of the fusion function in enveloped viruses is incompletely understood, we can only speculate on the relationship between altered fusion and reduced neuroinvasiveness. The altered fusion function could retard the initial steps in the infection of individual cells, particularly if endosomal vesicles in muscle cells have a higher average pH than do cells in the CNS. Alternatively, local tissue conditions (acidic pH during infection, for example) may allow for direct fusion of viruses to the plasma membrane. The higher pH at which LAC fuses membranes (pH 6.3) may be more easily attainable than the pH which activates the fusion function of V22 (pH 5.8), accounting for

TABLE 2. Frequency of brain lesions in mice infected with LAC or V22 virus

Age of mice	Virus	Dose in PFU (route)	Proportion of mice with specified lesions			
			Necrosis	Spongiform	Inflammation	Moderate or severe lesions ^a
Suckling	LAC	2,200 (i.p.)	3/3	0/3	0/3	3/3
	V22		1/3	1/3	0/3	1/3
Weanling	LAC	100,000 (i.p.)	6/14	8/14	5/14	6/14
	V22		4/10	3/10	6/10	2/10
Adult	LAC	1,000 (i.c.)	8/12	9/12	3/12	6/12
	V22		5/10	4/10	2/10	2/10
Total	LAC		17/29	17/29	8/29	15/29 ^b
	V22		10/23	8/23	8/23	5/23 ^b

^a Moderate and severe lesions were graded 3 or 4 on a scale of 1 to 4.

^b $P = 0.044$ (significant by chi-square test).

a greater proportion of infected cells. It is interesting that a similar correlation between fusion efficiency and peripheral virulence has been reported for mumps virus; neuroinvasive strains are better able to mediate cell-to-cell fusion than are nonneuroinvasive strains (24, 33).

Attenuated variants of other neurotropic viruses have been selected by using monoclonal antibodies (29, 30, 32; J. O. Fleming, S. A. Stohlman, and L. P. Weiner, manuscript in preparation). The attenuated variants of reovirus, rabies virus, and coronavirus differ in several ways from the V22 variant. First, they exhibit reduced neurovirulence on direct i.c. injection. Second, the mechanism of attenuation is different, since in the cases in which it has been studied (29, 30), there appears to be altered receptor binding. We demonstrated here that fusion, another important function of the external virion proteins, is also important in determining virulence.

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