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Genetic Basis for Altered Pathogenesis of an Immune-Selected Antigenic Variant of Reovirus Type 3 (Dearing)

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In this paper we provide a step by step comparison of the pathogenesis of murine infection caused by reovirus type 3 (Dearing) and an antigenic variant (K) selected by its resistance to neutralization with a monoclonal antibody (G5) directed against the T3 hemagglutinin. To show that specific changes in the biologic properties of variant K were due to mutation in the S1 double-stranded RNA segment (gene), which encodes the viral hemagglutinin, we generated a reassortant virus ("1 HA K") containing the variant K S1 gene and compared its properties to variant K and to a reassortant ("1 HA 3") containing the T3 (Dearing) S1 gene. These studies, in conjunction with our previous nucleotide sequence analysis of the S1 genes of variant K and T3 (Dearing) [R. Bassel-Duby, A. Jayasuriya, D. Chatterjee, N. Sonenberg, J. V. Maizel, Jr., and B. N. Fields, Nature (London) 315:421–423, 1985; R. Bassel-Duby, D. R. Spriggs, K. L. Tyler, and B. N. Fields, submitted for publication], indicate that a single amino acid change in the T3 hemagglutinin can alter viral growth and tropism within the central nervous system without affecting either its primary replication in the intestine or its pattern of spread to or within the central nervous system.

Antigenic variants of a number of neurotropic viruses have now been isolated. Many of these variant viruses have attenuated virulence (5, 6, 10, 14, 22, 26, 29, 35) resulting from defects in specific stages of pathogenesis, including primary replication (9, 14, 18) or central nervous system (CNS) tropism (22, 28).

The molecular and genetic basis for the altered biologic properties of neurotropic virus variants is only beginning to be understood. Apathogenic variants of rabies virus, selected by their resistance to neutralizing monoclonal antibodies directed against the surface glycoprotein (6), contain a nucleotide alteration in the glycoprotein gene. This change would result in the substitution of a new amino acid for the arginine normally present at position 333 in the glycoprotein (10, 26). A correlation between specific nucleotide substitutions and altered viral virulence has also been suggested based on sequence analysis of vaccine strains of poliovirus. Five base substitutions in the genome of poliovirus type 3 (P3/Leon/37) may be responsible for the attenuated phenotype of the Sabin vaccine strain (30), and reversion of the Sabin type 3 vaccine strain of poliovirus to a neurovirulent phenotype may correlate with a single nucleotide change in the 5′ noncoding region of the viral genome (11).

Our goal in this paper is to characterize the specific effects of a mutation involving the neutralization epitope of the reovirus T3 sigma 1 protein on virus-host interactions at each of a series of sequential stages during reovirus infection in vivo. First, we studied the nature of the altered biologic properties of a prototypic reovirus T3 sigma 1 antigenic variant (variant K). Then, to definitively establish that the variant K S1 gene was responsible for the altered properties of variant K, we created a reassortant virus containing the variant K S1 gene ("1 HA K") and compared its behavior in vivo to that of variant K. We also compared the properties of 1 HA K to those of an analogous reassortant containing the T3 S1 gene ("1 HA 3").

Since mutations can accumulate spontaneously at extremely high rates in the genomes of RNA viruses (15), and because we wished to definitively establish that the single nucleotide mutation present in the variant K S1 gene (1; R. Bassel-Duby, D. R. Spriggs, K. L. Tyler, and B. N. Fields, submitted for publication) was responsible for the altered biologic properties of variant K, we obtained the complete nucleotide sequence of the S1 gene of the reassortant 1 HA K to ensure that it was an exact copy of the variant K S1 gene.

These studies, in conjunction with our previous complete nucleotide sequence analysis of the variant K and type 3 (Dearing) S1 genes (1; Bassel-Duby et al., submitted), indicate that a single nucleotide change, resulting in a change in amino acid 419 (glutamic acid to lysine) of the 455-amino-acid reovirus sigma 1 protein, alters the pathogenicity of reovirus T3 by affecting its growth and tropism in the CNS without affecting the ability of the virus to enter the host via the gastrointestinal tract, replicate at its primary site in the intestine, or utilize its normal pathways of spread either to or within the CNS.

MATERIALS AND METHODS

Viruses. Reovirus type 1 Lang (T1) and type 3 Dearing (T3) were from standard laboratory stocks. The reassortant 1 HA 3 has been previously described; it contains a T3 S1 double-stranded RNA (dsRNA) segment with all other dsRNA segments derived from T1 (34). Reovirus variant K is a T3 variant selected with a neutralizing monoclonal antibody (G5) directed against the T3 sigma 1 protein (29). The reassortant 1 HA K was prepared for these experiments by using previously described procedures (24). Briefly, a confluent monolayer of cultured L strain fibroblasts (2.5×10^6 cells per 2 ml in a 2-dram [ca. 7.774 g] vial) was

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simultaneously coinfected with equal multiplicities (multiplicity of infection = 5) of T1 and variant K and then incubated at 31°C for 48 h. Input inoculum was then removed, and cells were incubated for an additional 48 h at 37°C. Infected cells were then lysed by three cycles of freezing (−70°C) and thawing (37°C). The cell lysate was then plated onto confluent L cell monolayers (2.5×10^6 cells per plate) in dilutions designed to produce well-isolated plaques in plastic petri dishes (60 by 15 mm). Plaques were picked according to previously outlined procedures (7). All clones were doubly plaque purified and then passaged twice on L cell monolayers (P2). These P2 stocks were used in all experiments. The parental origin of each dsRNA segment for the reassortant clones was established by performing 10% polyacrylamide slab gel electrophoresis of ^{32}P-labeled preparations of viral dsRNA by using previously described procedures (19, 23). Reassortant 1 HA K, isolated and identified by these methods, has an S1 dsRNA segment derived from variant K, with all other dsRNA segments derived from T1.

Neutralization assays. G5 is a neutralizing monoclonal antibody directed against the T3 sigma 1 protein (2). Neutralization tests were performed as previously described (2) by using G5 purified from supernatant fluid derived from cloned hybridoma cell cultures.

Animal inoculations. Pregnant CD-1 mice (Charles River Breeding Laboratories, Inc., N. Wilmington, Mass.) and pregnant NIH Swiss mice (National Cancer Institute, Frederick, Md.) were used for all experiments. Newborn mice were inoculated with virus within 48 h of birth. The technique for peroral (p.o.) inoculation of newborn mice has been previously described (25). Briefly, mice were anesthetized with methoxyflurane (Pitman-Moore), and a polyethylene catheter (0.58-mm inner diameter, 0.965-mm outer diameter, Intramedic 7510) was inserted into the stomach through the mouth. Virus (10^7 PFU) (0.050-ml volume) diluted in gelatin saline and mixed with blue food dye (0.06 ml/10 ml of gelatin saline, Durkee Famous Foods, Cleveland, Ohio) was inoculated through the catheter into the stomach. The dye was used as a marker to assess the accuracy of inoculation. For intracerebral (i.c.) and hindlimb footpad inoculations, 1- to 2-day-old mice were injected with 7.5×10^5 to 1.5×10^6 PFU of virus (i.c.) or 1.5×10^7 PFU of virus (footpad) in a 0.010-ml volume with a 30-g needle and a Hamilton syringe. For determination of i.c. 50% lethal doses (LD_{50}s), duplicate litters of 1- to 2-day-old mice were injected into the right cerebral hemisphere with either 10^2, 10^4, or 10^6 PFU of virus in a 0.030-ml volume with a 26-g needle and a tuberculin syringe. Animals were observed daily for 2 weeks for mortality.

Titration of virus in mouse organs. Mice were sacrificed by either decapitation or cervical dislocation. Organs were removed by using sterile techniques, placed in 1 ml of gelatin saline, and stored at −70°C. Specimens of intestine included the entire small bowel from the gastroduodenal junction to the ileocecal valve. Intestinal tissues were washed three times in cold phosphate-buffered saline before being placed in gelatin saline and stored at −70°C. Spleen specimens included the entire spleen. Brain specimens included both cerebral hemispheres, the cerebellum, and the brain stem down to the level of the cervico-medullary junction. Superior spinal cord (SSC) specimens included the entire cervical spinal cord and one-third to one-half of the upper thoracic cord. Inferior spinal cord (ISC) specimens included the lower thoracic cord and the entire lumbosacral cord. Spinal cord specimens were obtained, after dissecting out the vertebral column en bloc, by pressure extrusion of cord tissue from the surrounding vertebral column. Eye specimens included the entire globe.

All specimens were frozen (−70°C) and thawed (37°C) three times and then sonicated for 15 s (brain, SSC, ISC) or 30 s (eye, spleen, intestine) (Ultrasonics W-225R sonicator, microtip probe, output setting 3.5). The tissue specimens were then serially diluted in gelatin saline and plated in duplicate onto confluent L cell monolayers in Costar 6-well plates (Costar, Cambridge, Mass.). Plaque assay techniques were as previously described (13, 25).

Pathologic examination. For pathologic examination, brains were fixed in 10% (wt/vol) buffered Formalin (Fisher Scientific Co., Pittsburgh, Pa.) and then embedded in Paraplast (Sherwood Medical Industries). Coronal sections were obtained and stained with hematoxylin and eosin by using standard histologic techniques (4).

Nucleotide sequence analysis. The S1 dsRNA was sequenced as described in the procedure of Bassel-Duby et al. (submitted). Briefly, genomic RNA was obtained from purified virus as previously described and electrophoresed on a 10% polyacrylamide electrophoresis gel. The S1 dsRNA segment was electroeluted from the gel and denatured by incubation in 90% dimethyl sulfoxide in 1 mM Tris hydrochloride (pH 6.8) at 50°C for 45 min. The RNA was incubated with synthetic primers and directly added to a reaction mix with the appropriate deoxynucleotide and dideoxynucleotide concentrations, ^{35}S-labeled dATP, reverse transcriptase, and RNasin. The sequencing reaction was carried out at 37°C for 30 min. The reaction was chased with deoxynucleotides, and formamide-dye mix was added. The samples were boiled for 3 min and loaded onto an 8% sequencing gel.

RESULTS

In our experiments we wished to systematically examine the behavior of variant K and compare it to wild-type T3 (Dearing) at each of a series of sequential steps during viral pathogenesis. To establish the role of the K S1 gene in altering the biologic properties of variant K, we also examined the behavior of a reassortant virus (1 HA K) containing the variant K S1 gene. We expected that the behavior of 1 HA K would not be identical to that of variant K because of the presence in the reassortant of nine reovirus T1 genes (25). We therefore compared 1 HA K with a reassortant, 1 HA 3, with which it is identical except that 1 HA 3 contains the T3 S1 gene rather than the variant K S1 gene.

Characterization of 1 HA K. Before beginning our studies of viral pathogenesis, we wished to ensure that the reassortant 1 HA K contained an exact copy of the variant K S1 gene. Accordingly, we sequenced the S1 gene from 1 HA K by using primer extension techniques (Bassel-Duby et al., submitted). We then compared the S1 gene nucleotide sequence of 1 HA K to those of variant K and T3 (Dearing). The nucleotide sequences of the S1 genes of 1 HA K and variant K were identical, and both differed from that of T3 (Dearing) in containing a single nucleotide change (G → A at position 1267), resulting in a substitution of a lysine for a glutamic acid at position 419 of the sigma 1 protein. Thus, sequencing the S1 gene confirmed that the process of reassortment and subsequent passage in L cells did not result in either the introduction of additional nucleotide alterations or the reversion of the variant K S1 gene to the T3 nucleotide sequence.

We next wished to establish that the sigma 1 protein (hemagglutinin) of 1 HA K was identical to that of variant K.

Variant K was selected by its resistance to neutralization with a monoclonal antibody (G5) directed against the neutralization (NT) epitope of the T3 (Dearing) sigma 1 protein (29). 1 HA K, like variant K, and unlike T3, was also resistant to neutralization with G5 (Table 1). This indicated that the NT epitope of the 1 HA K sigma 1 protein was similar to that of the variant K sigma 1 protein but differed from the NT epitope of the T3 (Dearing) sigma 1 protein.

Thus, the results obtained by nucleotide sequence analysis and neutralization studies indicated that 1 HA K contained an exact copy of the variant K S1 gene and contained a sigma 1 protein of the variant K type.

Growth of virus in intestinal tissue. We began our experiments on viral pathogenesis by studying the growth of virus in intestinal tissue after p.o. inoculation of 10^7 PFU of virus into 2-day-old mice. Since reoviruses are enteric viruses, p.o. inoculation mimics a natural route of entry for which the intestine is the site of primary viral replication. After p.o. inoculation, variant K reached a peak titer in the intestine of 10^6 PFU at 12 h postinoculation, which subsequently declined over the next 72 h. Titers for T3 were initially (12 h) below those for variant K but were similar at 24 and 72 h (Fig. 1).

We previously suggested that reassortant reoviruses containing certain reovirus T1 genes have an enhanced capacity to grow in the intestine after p.o. inoculation (25). We therefore anticipated that the reassortant 1 HA K would grow to a higher titer in intestinal tissue than would variant K, but that this difference would be similar to that seen when the reassortant 1 HA 3 was compared with T3.

After p.o. inoculation, 1 HA K reached a peak titer of $\sim 10^7$ PFU. This level was similar to that seen with T1 and 1 HA 3 (Fig. 1). Thus, these experiments indicated that variant K and T3 did not differ significantly in their pattern of primary replication after entering the host via their natural enteric pathway and that the growth of variant K in intestinal tissue, like that of T3, was enhanced by the presence of T1 genes.

Spread of virus to the brain after p.o. inoculation. Since replication at the primary site did not appear to differ significantly between variant K and T3, we examined the capacity of these viruses to spread to the CNS after p.o. inoculation. After p.o. inoculation of 10^7 PFU of T3 into 2-day-old mice, virus spread to both the spleen and brain (Fig. 2). Virus was not detected in the brain before day 6, and peak brain titers (10^6 PFU/ml) were reached on day 9.

TABLE 1. Neutralization of T3, variant K, 1 HA 3, and 1 HA K with G5 monoclonal antibody

Virus	% of plaques observed after incubation with following dilution of G5[a]:					
	1:40	1:200	1:500	1:1,000	1:2,500	1:5,000
T3	3	7	7	18	20	23
K	100	100	100	100	100	100
1 HA 3	7	14	15	22.5	25	27
1 HA K	100	100	100	100	100	100

[a] Neutralization assays were performed as previously described (2). Purified monoclonal antibody G5 was diluted as indicated in minimal essential medium. A total of 200 PFU of the indicated virus was added to the diluted antibody and incubated at 37°C for 1 h. Samples were then plated onto triplicate wells of 6-well Costar dishes and allowed to adsorb at 37°C for 1 h, and then standard reovirus plaque assay procedures were followed (13). Values represent the percentage of plaques observed after incubation with the indicated dilution of G5 compared with the number of plaques present when the indicated virus was incubated with phosphate-buffered saline.

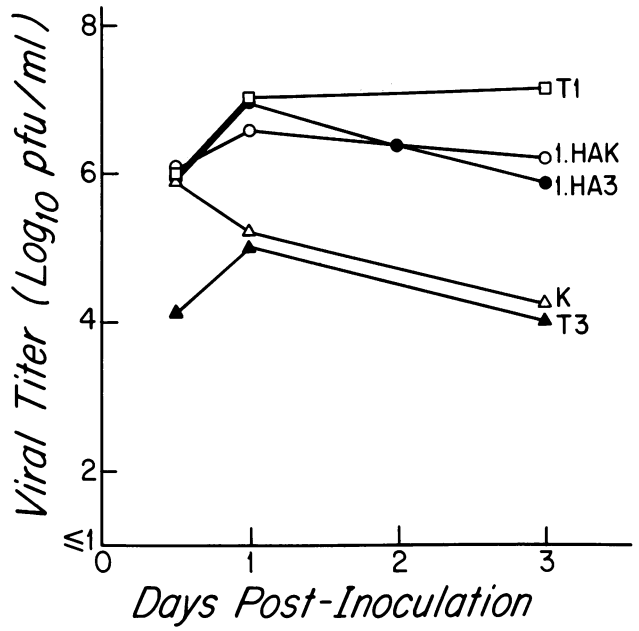

FIG. 1. Titers of T1, T3, variant K, 1 HA K, and 1 HA 3 in intestinal tissue after p.o. inoculation of 10^7 PFU into 2-day-old NIH(S) mice. Each point represents the average of the $\log_{10}$ titers of at least three specimens.

After p.o. inoculation, variant K spread to the spleen like T3 but could not be detected in the brain (Fig. 2).

We then compared the spread of 1 HA K from the gastrointestinal tract with that of variant K and 1 HA 3 (Fig. 3). 1 HA K was able to spread to the brain as well as to the spleen after p.o. inoculation. When its pattern of spread was compared to that of 1 HA 3, several differences were apparent. 1 HA 3 was detected in the brain 2 days sooner than 1 HA K. Once 1 HA 3 reached the brain, it grew

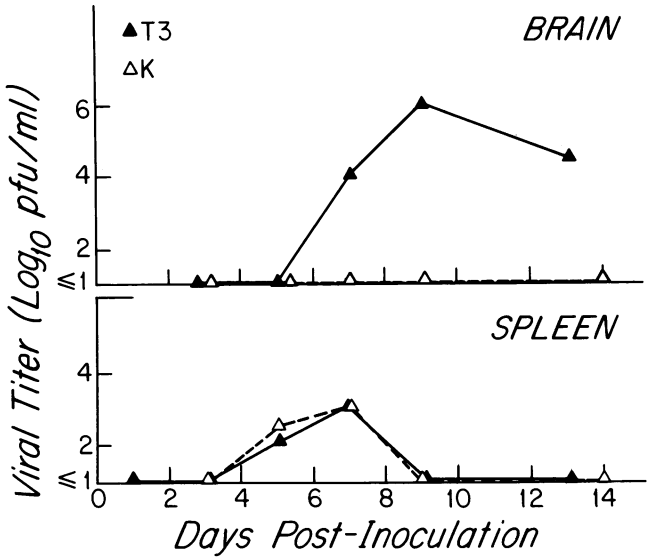

FIG. 2. Titers of T3 and variant K in the brain and spleen of NIH(S) mice after p.o. inoculation of 10^7 PFU of virus. Mice were 2 days old at the time of initial inoculation. Each point represents the average of the $\log_{10}$ titer of organs obtained from at least three animals.

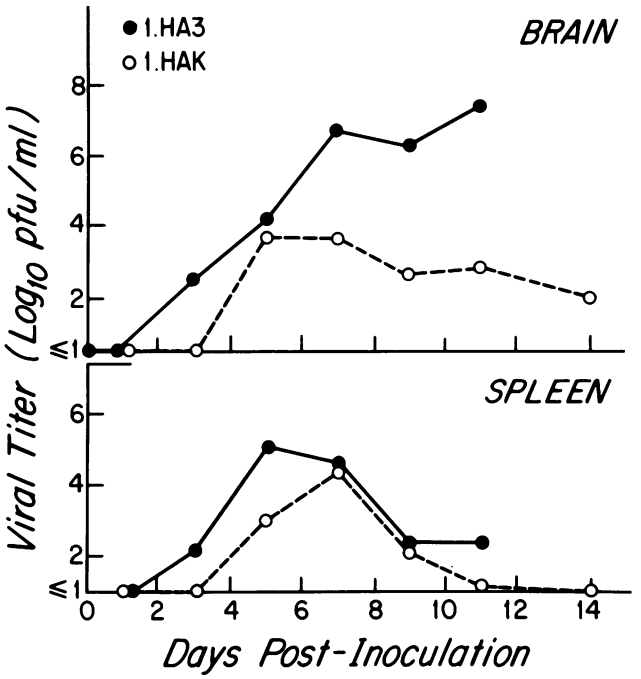

FIG. 3. Titers of 1 HA 3 and 1 HA K in the brain and spleen of NIH(S) mice after p.o. inoculation of 10^7 PFU into 2-day-old mice. Each point represents the average of the $\log_{10}$ titers obtained for at least three mice.

rapidly, reaching a peak titer 4 $\log_{10}$s higher than that achieved by 1 HA K.

Spread of virus to the CNS. The results described above suggested that variant K and T3 differed in their capacity to spread to the CNS after p.o. inoculation. Since the pathways used by reoviruses to spread from the gastrointestinal tract to the CNS are not known, we decided to study the spread of virus to the CNS by using an experimental system which allowed us to differentiate between hematogenous and neural pathways of spread to the CNS (K. L. Tyler, D. McPhee, and B. N. Fields, Science, in press). Virus was inoculated into the hindlimb footpad of 1-day-old mice, and at subsequent time points the amount of virus present in the superior and inferior spinal cord was assayed. If virus reached the spinal cord through neural pathways (neural spread), then the titer of virus would be significantly higher in the spinal cord block containing the neurons innervating the skin and muscle at the injection site. Hematogenous spread of virus would result in the appearance of virus throughout the spinal cord at the same time and in equivalent amounts.

After hindlimb footpad inoculation of variant K, viral titer was consistently higher in the ISC than in the SSC (Fig. 4). This pattern was similar to that for T3 (Tyler et al., in press) and indicated that variant K reached the spinal cord by neural spread. Thus, the mutation present in variant K did not qualitatively alter its capacity for neural spread.

Our previous studies on the pattern of spread of T1 indicated that T1 spread to the spinal cord via a nonneural, presumably hematogenous route (Tyler et al., in press). Studies with reassortant viruses indicated that the T3 S1 gene was primarily responsbile for determining the capacity of reovirus to spread to the spinal cord via nerves (Tyler et al., in press). Thus, the reassortant 1 HA 3, which contains the T3 S1 gene and all other genes from T1, spreads via nerves. To see whether the variant K S1 gene was capable of determining a neural spread pattern, we studied the spread of 1 HA K to the spinal cord after footpad inoculation. After hindlimb footpad inoculation, 1 HA K reached the ISC in significantly higher titers than it did the SSC (Fig. 4). Thus, the presence of the variant K S1 gene did not qualitatively alter the pathways of spread used by either variant K or 1 HA K to reach the CNS, and the variant K S1 gene retained the capacity to determine neural spread.

Growth of virus within the CNS. The growth of variant K in the brain was attenuated compared with that of T3 (29) (Fig. 5). When the reassortant 1 HA K reached the brain after p.o. inoculation, its growth was also strikingly attenuated, especially when compared with that of 1 HA 3 (Fig. 3). This strongly suggests that the variant K S1 gene is responsible for the attenuated CNS growth of variant K. To establish this directly, we compared the growth of 1 HA K after i.c. inoculation with that of 1 HA 3. 1 HA K showed markedly attenuated growth in the brain when compared with 1 HA 3 (Fig. 5), indicating that attenuated growth was indeed due to the variant K S1 gene.

Tropism of virus in the CNS. Inoculation of T3 into the brain of neonatal mice results in the development of a necrotizing encephalitis which predominantly involves the cingulate gyrus, striate cortex, hippocampus, and septal

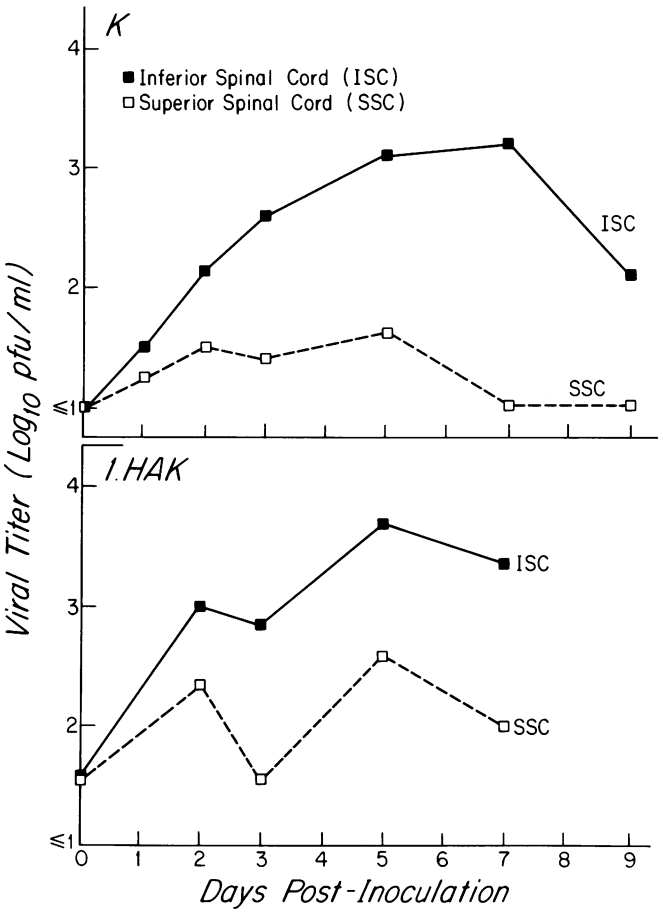

FIG. 4. Titers of variant K and 1 HA K in the ISC or SSC of NIH(S) mice injected subcutaneously with 8.5×10^5 to 2×10^6 PFU of virus into the hindlimb footpad. Mice were 1 day old at the time of inoculation. Each point represents the average of the $\log_{10}$ titers of specimens taken from five mice. Maximum standard deviation at any time point was <0.5 $\log_{10}$.

areas. The distribution of CNS injury produced by variant K differs from that of T3, because variant K does not produce cortical damage (28). To determine whether the pattern of CNS injury produced by the reassortant 1 HA K was identical to that produced by variant K, we injected 10^6 PFU of 1 HA K i.c. into 1-day-old mice and examined hematoxylin and eosin-stained sections of Formalin-fixed brains obtained 8 to 14 days later. Lesions were identical to those previously described for variant K (data not shown).

Spread of virus within the CNS. We wished to test whether variant K had an altered pattern of spread within the CNS. We showed that after i.c. inoculation, T3 is able to spread to the eye and infect retinal ganglion cells (31). Because the retina is directly connected to the brain via the optic nerve, this provides a model for studying intra-CNS spread of virus.

Variant K was able to spread to the eye after i.c. inoculation (Fig. 6). The peak viral titer reached by variant K in the eye was 3.5 $\log_{10}$s lower than that achieved by T3. When the reassortant 1 HA K was inoculated into the brain it also reached the eye, and its peak titer was 2.5 $\log_{10}$s lower than that achieved by 1 HA 3. In contrast to T3 and 1 HA 3, variant K and 1 HA K both showed a pronounced decrease in titer after their initial peak. This growth pattern is similar to that seen in the brain itself after i.c. inoculation (Fig. 5) (29). Thus, both variant K and the reassortant 1 HA K showed a qualitatively normal pattern of intra-CNS spread in this system, but once they reached the retina they continued to show a pattern of attenuated growth.

Neurovirulence. We previously reported that the i.c. LD_{50} of variant K for neonatal mice was more than 6 $\log_{10}$s greater than that for T3 (Dearing) (29). We therefore wished to determine whether 1 HA K would also show markedly attenuated neurovirulence when compared with 1 HA 3 (Fig. 7). All (100%) 1-day-old mice inoculated i.c. with 10^2, 10^4, or 10^6 PFU of 1 HA 3 died (LD_{50} < 100 PFU). Conversely, when 10^2 or 10^4 PFU of 1 HA K was inoculated i.c. there were no deaths, and when 10^6 PFU was inoculated there was only 30% mortality (LD_{50} > 10^6 PFU). These results indicate that the variant K S1 gene was responsible for the attenuated neurovirulence of variant K.

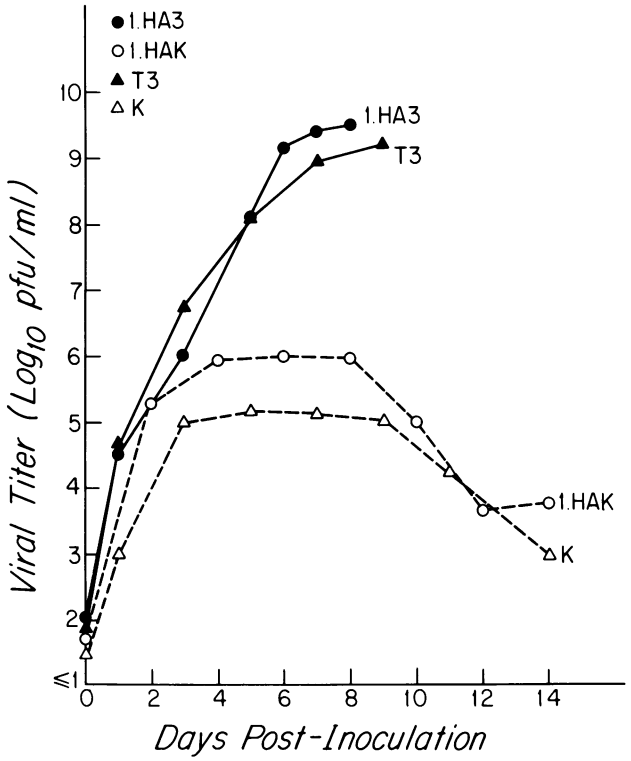

FIG. 5. Titers of T3 and variant K or 1 HA 3 and 1 HA K in the brain after inoculation of 10^3 PFU of virus into the right cerebral hemisphere of 1-day-old NIH(S) mice. Each point represents the average of the $\log_{10}$ titers of five specimens (K and 1 HA K) or eight specimens (T3 and 1 HA 3). Maximum standard deviation at all time points was <1.0 $\log_{10}$ for K and 1 HA K or <0.5 $\log_{10}$ for T3 and 1 HA 3.

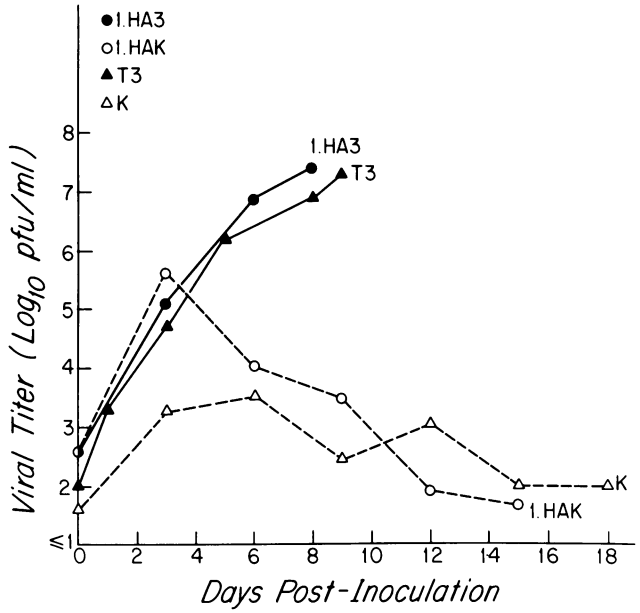

FIG. 6. Titers of T3 and variant K or 1 HA 3 and 1 HA K in the eye after i.c. inoculation of 1×10^6 to 3×10^6 PFU of virus into 1-day-old NIH(S) mice. Each point represents the average of the $\log_{10}$ titers of 10 eyes (five mice). Maximum standard deviation at all time points was <0.5 $\log_{10}$ for T3 and 1 HA 3 or <0.75 $\log_{10}$ for K and 1 HA K.

DISCUSSION

In these studies we have attempted to (i) further expand our understanding of the functions of the reovirus sigma 1 protein by clearly defining the nature of the altered pathogenic properties of the reovirus sigma 1 antigenic variant K and (ii) unequivocally establish that these properties are the result of a single nucleotide change in the viral S1 gene which results in the substitution of a lysine for a glutamic acid at amino acid position 419 in the viral sigma 1 protein.

Because previous studies with antigenic variants of neurotropic viruses suggested several possible explanations for their altered pathogenicity (9, 14, 18, 22), a major goal of our current studies was to characterize the behavior of variant K and the reassortant 1 HA K at each of a variety of specific stages in pathogenesis.

Variant K and T3 showed similar growth patterns in the intestine after p.o. inoculation, as did 1 HA K and 1 HA 3. These results indicated that defective primary replication did not account for the altered pathogenicity of variant K after p.o. inoculation. We then studied the spread of virus from the intestine and found that although variant K was able to spread to the spleen in a fashion similar to T3, unlike T3, we

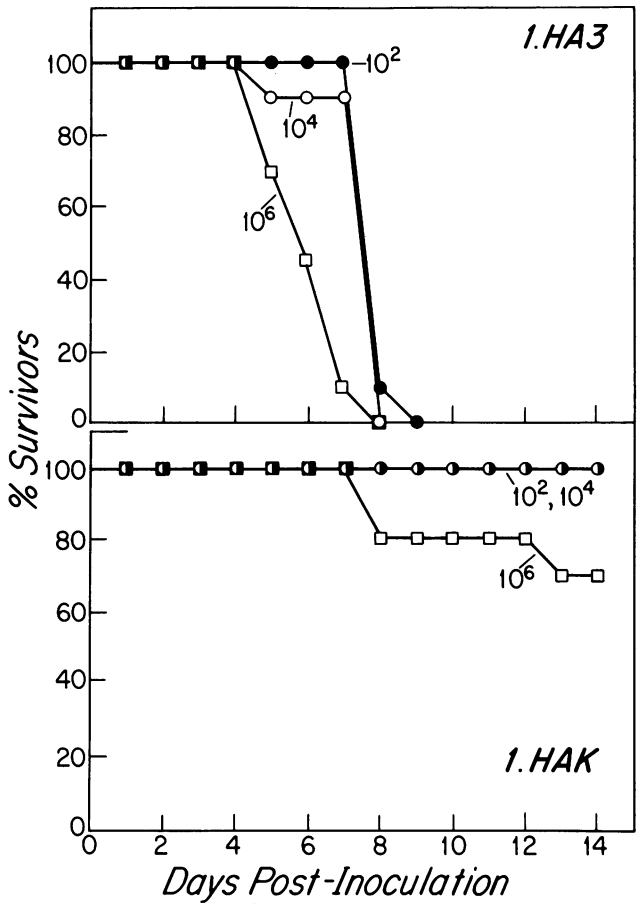

FIG. 7. Survival of 1- to 2-day-old mice [NIH(S) or CD-1] after i.c. inoculation of the indicated doses of 1 HA 3 or 1 HA K. Each curve represents the results obtained with at least two litters of mice. Mice were checked daily for 2 weeks for deaths.

were unable to detect variant K in the brain after p.o. inoculation. This suggests that the requirements for spread of reovirus from the gut to the spleen differ from those required for spread to the CNS. In adult C3H/HeJ mice, reovirus T1 spreads sequentially from the intestine to Peyer's patches, mesenteric lymph nodes, and then the spleen (16). The pathway of spread used by reovirus T3 to reach the spleen is not known, but our data indicate that the capacity of reovirus T3 to reach the spleen is not altered by the mutation in the variant K S1 gene.

Variant K was not detected in the brain after p.o. inoculation. This may have been due to either defective spread of variant K or failure of variant K to achieve detectable levels of replication once it reached the brain. This second possibility does not appear likely in light of the sensitivity of our assays (detection of as little as 10 PFU per brain).

Unlike variant K, the reassortant 1 HA K was detected in the brain after p.o. inoculation. This suggests either that the defective spread of variant K is not due to the S1 gene or that the presence of reovirus T1 gene(s) in 1 HA K may enable this reassortant to overcome the effect of the variant K S1 gene on spread. We previously showed that the capacity of certain reovirus reassortants to spread to the CNS correlates with their enhanced capacity to grow in the intestine and that both of these properties are due to the presence of certain reovirus T1 genes (25). 1 HA K, which contains nine type 1 genes, in addition to the variant K S1 gene, shows both the capacity to spread to the CNS and enhanced intestinal growth when compared to variant K. These results suggest that the type 1 genes present in 1 HA K may allow this variant to overcome the spread effect of the variant K S1 gene, perhaps by enhancing the intestinal growth of 1 HA K. Some minimum level of primary replication in the intestine may be required before reoviruses enter neural or other spread pathways.

Unfortunately, the precise pathways by which reoviruses spread from the intestine to the CNS have not yet been identified. Therefore, to further study the spread of variant K to the CNS we used another experimental system in which the pathways of spread to the CNS are better defined. Our previous work indicated that T3 is dependent primarily on neural pathways to reach the spinal cord after peripheral inoculation into either the forelimb or hindlimb footpad (Tyler et al., in press). We found that both variant K and 1 HA K, like their counterparts T3 and 1 HA 3, also utilized neural pathways of spread to reach the spinal cord (Fig. 4). These results indicate that the mutation present in the variant K S1 gene did not alter the capacity of the virus to utilize its normal neural pathway of spread. Our results did not allow us to determine whether variant K had an altered rate of spread when compared to T3. It did appear, when the spread of 1 HA 3 and the spread of 1 HA K from the gastrointestinal tract to the brain were compared, that 1 HA K reached the brain later than 1 HA 3. If 1 HA K does have delayed spread, this could conceivably occur either at the level of entry into neural pathways of spread or during the process of neural spread itself.

Defects in the capacity to spread to the CNS may be present in other immune-selected variant viruses. Some of the antigenic variants of rabies virus appear to be able to utilize only specific subsets of the neural pathways available to the wild-type virus (18). In the case of La Crosse bunyavirus variant V22, decreased "neuroinvasiveness" seems to be due to poor replication at the primary site (14). A connection between the degree of primary replication and spread is also suggested by our experiments, in which the reassortant 1 HA K showed both enhanced intestinal growth and facilitated CNS spread when compared with variant K.

In addition to studying the spread of virus from peripheral tissues to the CNS, we studied the spread of virus from the brain to the eye to see whether there was any evidence for defective spread of virus within the CNS. Some rabies virus variants appear to have a decreased rate of spread within the CNS (9). Both variant K and 1 HA K appeared to be capable of spreading from the brain to the retina with the same facility as their counterparts T3 and 1 HA 3 (Fig. 6), suggesting that intra-CNS spread is not qualitatively altered in variant K.

The predominant differences between variant K and T3 appear to lie in the pattern of growth and tropism in the CNS. We believe that the attenuated growth and altered tropism of variant K account for its markedly diminished neurovirulence compared with that of T3 (Dearing). When the reassortant 1 HA K reached the brain, either by spreading from the gastrointestinal tract or after direct i.c. inoculation, it showed the pattern of attenuated growth, altered CNS tropism, and decreased virulence characteristic of variant K. These effects are particularly obvious when the CNS growth patterns (Fig. 3 and 5) and LD_{50} data (Fig. 7) for 1 HA K and 1 HA 3 are compared. Attenuated growth does not appear to be confined to the brain, since both variant K and 1 HA K show an attenuated growth pattern in other

parts of the CNS, including the spinal cord and retina. These results clearly establish that the variant K S1 gene is responsible for the attenuated neurovirulence, diminished CNS growth, and altered CNS tropism of variant K and the reassortant 1 HA K.

In addition to providing a comprehensive analysis of the biologic properties of variant K during in vivo infection, a second major goal of our studies was to try to definitively establish that the specific alteration in the S1 gene of variant K was responsible for specific changes in viral properties. We particularly wished to establish that the variant K S1 gene alteration was not merely coincidental or simply a marker for the altered properties of variant K.

Mutations accumulate spontaneously in the genomes of RNA viruses at an extremely high rate (15). This makes the problem of correlating identified gene alterations with specific changes in the biologic properties of a virus difficult. Recent comparisons of the nucleotide sequences of the neuraminidase genes of an avirulent avian influenza virus and its virulent derivative illustrate the nature of this problem. During a period of 6 months, eight nucleotide substitutions appeared in this single gene (8). Obviously in this situation it would be extremely difficult to decide which of these mutations might have resulted in the altered virulence of the virus.

An indirect argument can be made that a particular gene alteration does in fact determine a specific biologic property if it can be shown that the change is the only one present and that it consistently and invariably accompanies a particular phenotypic alteration. In the case of immune-selected variant viruses it may be difficult to establish that only one gene alteration is present without performing complete sequence analysis of the genomes of both the wild-type and variant viruses. Although immune-selected influenza virus variants are typically found to have single amino acid substitutions in the protein against which the selecting monoclonal antibody is directed (3, 17, 20, 21, 32, 33, 36), some immune-selected variants of poliovirus and rhinovirus have been found to have mutations involving two proteins (12, 27).

Our previous studies established the complete nucleotide sequence for the S1 gene in both variant K and T3 (Dearing) (1; Bassel-Duby et al., submitted). Having found that only a single nucleotide change was present in the S1 gene, we wished to prove that this change was responsible for the altered properties of variant K. We therefore generated a reassortant virus containing the S1 gene of variant K and all other genes derived from reovirus type 1 (T1). We compared the behavior of this reassortant to that of variant K by using a variety of experimental systems designed to evaluate viral primary replication, spread, CNS growth, and tropism. In order to identify any possible modulating effect of the T1 genes in the reassortant 1 HA K, we compared the behavior of 1 HA K to that of an analogous reassortant (1 HA 3), which contains the T3 S1 gene in place of the variant K S1 gene. We also sequenced the S1 gene present in the reassortant 1 HA K to be sure that it was identical to that of variant K and that the process of reassortment had not resulted in either reversion or introduction of novel additional mutations.

We believe that our studies of variant K and the reassortant 1 HA K, in conjunction with our previous sequence analysis of the S1 genes of variant K and T3, provide a comprehensive picture of the effect of mutation in the NT epitope of the T3 sigma 1 protein on the interaction between a virus and its host. We have shown that variant K is defective at specific steps in pathogenesis, including CNS growth and tropism. Neither its pattern of primary replication in the gut nor its pattern of spread either to or within the CNS was altered. Our studies with the reassortant 1 HA K and our previous sequence analysis of the wild-type and variant K S1 genes enable us to conclude that the specific alterations in the pathogenic phenotype of variant K that we have identified are determined by a single nucleotide change in the S1 gene which results in a single amino acid substitution (lysine for glutamic acid at position 419) in the viral sigma 1 protein.

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