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Molecular basis of reovirus virulence: Role of the S1 gene

(cell tropism/neurovirulence/ependyma and neurons/reovirus recombinants)

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ABSTRACT **A genetic approach has been used to define the molecular basis for the different patterns of virulence and central nervous system cell tropism exhibited by reovirus types 1 and 3. Intracerebral inoculation of reovirus type 3 into newborn mice causes a necrotizing encephalitis (without ependymal damage) that is uniformly fatal. Animals inoculated with reovirus type 1 generally survive and may develop ependymal cell damage (without neuronal necrosis) and hydrocephalus. Using recombinant clones derived from crosses between reovirus types 1 and 3, we have been able to determine that the S1 genome segment is responsible for the differing cell tropism of reovirus serotypes and is the major determinant of neurovirulence. The type 1 S1 genome segment is responsible for ependymal damage with subsequent hydrocephalus; the type 3 S1 genome segment is responsible for neuronal necrosis and neurovirulence. We postulate that these differences are due to the specific interaction of the σ1 outer capsid polypeptide (the protein coded for by the S1 genome segment) with receptors on the surface of either ependymal cells or neuronal cells.**

The unique specificities of individual viruses for certain host species, as well as different tissues within a host, are hallmarks of viral infections. The molecular basis of these properties is poorly understood.

The different serotypes of reovirus [defined on the basis of neutralizing and hemagglutination inhibition antibodies (1, 2)] exhibit differing tropisms for cells in the central nervous system and, presumably as a consequence of their differing tropisms, produce differing patterns of virulence. Specifically, when reovirus type 3 is inoculated intracerebrally into newborn mice, an acute encephalitis develops that is fatal in virtually 100% of animals and is accompanied by destruction of neuronal cells without damage to ependymal cells (3, 4). Intracerebral inoculation of reovirus type 1 produces a nonfatal infection involving the ependymal cells that line the ventricular cavities of the brain, with little or no effect on neurons (5). Hydrocephalus often develops as a sequel to the ependymal damage (6). The basis for this difference in virulence and cell tropism between reovirus types 1 ("avirulent") and 3 ("virulent") is unknown.

Reoviruses are animal viruses whose genomes consist of 10 segments of double-stranded RNA (dsRNA) named according to size classes: 3 large segments (L1, L2, L3), 3 medium segments (M1, M2, M3), and 4 small segments (S1, S2, S3, S4). The dsRNA is surrounded by a double capsid shell, the outer capsid consisting of three polypeptides, σ1, σ3, and μ2, derived from genome segments S1, S4, and M2, respectively (7, 8).

Using recombinant clones derived from crosses between reovirus types 1 and 3, and consisting of genome segments derived from both parents, we have previously shown that the S1 gene, the dsRNA segment that codes for the σ1 outer capsid polypeptide, is responsible for type specificity [as determined by neutralization (9)] and for hemagglutination (H. L. Weiner, unpublished observations). Using the same genetic approach, we have been able to determine that the S1 dsRNA segment is the gene responsible for the differing cell tropisms of reovirus serotypes and is a major determinant of neurovirulence.

MATERIALS AND METHODS

Cells. Mouse L cells were maintained in suspension culture in Joklik's modified Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 5% fetal calf serum [International Biological Laboratories (IBL), Rockville, MD]. Monkey CV-1 cells were maintained in monolayers in IMEMZO ("improved minimal essential media supplemented with zinc, insulin and Hepes buffer," IBL) supplemented with 10% fetal calf serum (IBL).

Virus. Reovirus type 1 (Lang strain) and type 3 (Dearing strain) were the same as previously described (10). Recombinant clones were prepared by mixedly infecting L cells with equal multiplicities of temperature-sensitive (ts) mutants of type 3 reovirus (11) and clones were collected at 39° (a nonpermissive temperature for the temperature-sensitive mutants) and shown to be recombinants.§ Eight such clones were utilized in the present analysis (Fig. 1 and Table 1). The origin of these clones is described in detail elsewhere.¶

Analysis of Viral RNA. Viral RNA was analyzed as previously described (10). Briefly, CV-1 monolayers were inoculated with the two serotypes of reovirus or hybrid clones at a multiplicity of infection of 10 and were grown at 31° on 60- × 15-mm plastic Falcon tissue culture dishes (Falcon, Oxnard, CA) in 5 ml of IMEMZO. At 2.5 hr after infection, medium containing 0.25 mCi of ^{32}P was added. At 48 hr, infected monolayers were washed with an isotonic buffer (140 mM NaCl/10 mM Tris·hydrochloride at pH 7.4/1.5 mM $MgCl_2$; buffer A) and cells were collected. The cells were treated with 0.5% Nonidet P-40 in buffer A, nuclei were removed by centrifugation, and RNA was precipitated at −20° in ethanol. The precipitates were pelleted by high-speed centrifugation, dried, dissolved in gel sample buffer, and applied to slab gels (10% acrylamide/0.267% bisacrylamide) as described by Laemmli (12). After electrophoresis, gels were fixed and autoradiography was performed.

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Abbreviations: dsRNA, double-stranded RNA; PFU, plaque-forming units.

§ Sharpe, A. H., Ramig, R. F., Mustoe, T. A. & Fields, B. N., "A genetic map of reovirus. I. Correlation of genome RNAs between serotypes 1, 2, and 3," *Virology*, in press.

¶ Ramig, R. F., Mustoe, T. A., Sharpe, A. H. & Fields, B. N., "A genetic map of reovirus. II. Assignment of the double-stranded RNA negative mutant groups C, D, and E to genome segments," *Virology*, in press.

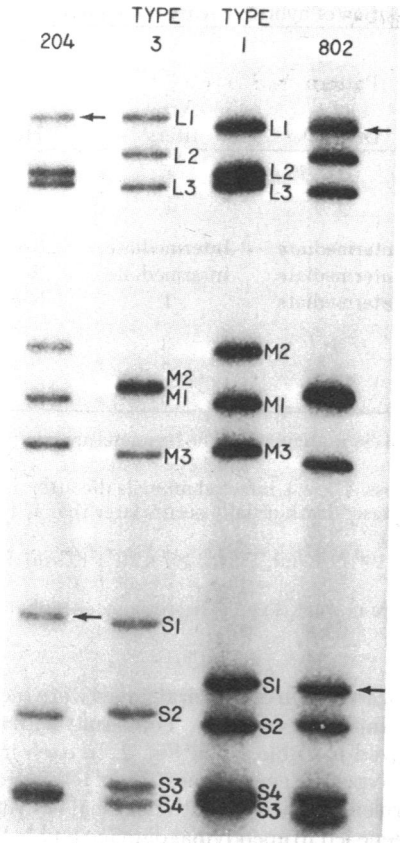

FIG. 1. Autoradiograms of reovirus dsRNA segments from serotypes 1 and 3 and hybrid clones 204 and 802, electrophoresed in a Tris/glycine gel system (10). The three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) genome segments of serotypes 1 and 3 are labeled. Each of the genome segments from the two serotypes can be distinguished on the basis of migrational differences. The numbering of the medium and small segments is in accordance with numbering of these segments in Tris/acetate gel systems. Clone 204 contains genome segments L1 and S1 (arrows) from type 3 and the remainder of segments from type 1. Clone 802 contains genome segments L1 and S1 (arrows) from type 1 and the remainder of segments from type 3.

Inoculation of Animals and Viral Titrations. Pregnant BALB/c mice were obtained from Charles River Laboratories, North Wilmington, MA, and newborns were inoculated intracerebrally within 24 hr of birth with various amounts of virus [3×10^5 to 3×10^2 plaque-forming units (PFU)/mouse] in a volume of 0.03 ml using a tuberculin syringe with a 26-guage needle. Triplicate litters (a total of 15–20 animals) were inoculated with each clone or parental serotype at each dilution of virus. Survival curves were determined by recording the number of animals alive up to 15 days after inoculation. Animals dying on day 1 or 2 after inoculation (approximately 4% of animals inoculated) were not included in survival curves. Animals were observed twice daily and the brains from dead animals were either fixed in formalin for routine hematoxylin/eosin pathologic analysis or frozen at −70° for determination of viral titers in brain tissue. Surviving animals were sacrificed at day 15; occasionally, healthy animals were sacrificed at day 7. All viral titrations of brain tissues were done on animals injected with 3×10^5 PFU. For viral titrations, brains were thawed, added to 2 ml of gelatin saline (to make a 10% suspension), and sonicated. Cell debris were removed by low speed centrifugation (600g for 15 min), and the virus-containing supernatant was then assayed on L cell monolayers according to the plaque assay of Fields and Joklik (11).

RESULTS

Disease Pattern in Animals Injected with Reovirus Type 3. We have confirmed the finding that type 3 reovirus, when injected intracerebrally into newborn mice, is uniformaly fatal at doses ranging from 3×10^5 PFU injected/mouse to 3×10^3 PFU injected/mouse; at 3×10^2 PFU/mouse 20% of the animals survive (3, 4). At higher doses (3×10^5) animals become sick at day 5–6 and are dead by day 8. At lower doses (3×10^2) animals become sick at day 8–9 and most are dead by day 10–12 (Fig. 2). Histological sections of the brains of animals dying following intracerebral injection of type 3 reovirus show a necrotizing encephalitis with destruction of neuronal cells manifest by fragmentation of the nucleus and eosinophilic cytoplasm. The ependymal cells that line the ventricular cavities of the brain are unaffected (Fig. 3). Viral titers of brains from dead animals that were injected with 3×10^5 PFU of type 3 reovirus were consistently greater than 5×10^9 PFU/ml.

Disease Pattern in Animals Injected with Reovirus Type 1. In contrast to the lethal effects of reovirus type 3, the majority of newborn mice injected with type 1 reovirus at doses ranging from 3×10^5 PFU injected/mouse to 3×10^3 PFU injected/mouse survive (Fig. 2). Some of the animals injected with type 1 reovirus show necrosis of the ependymal cells that line the ventricular cavities of the brain and hydrocephalus (Fig. 3); many animals show normal histologic patterns. In contrast to type 3 reovirus, there is no involvement of neuronal cells. Thus, the sporadic deaths seen in animals injected with type 1 are apparently not due to the same mechanism as that seen with type 3, because there is no neuronal involvement. In fact, the brains of such animals are histologically normal. The viral titers in brains of animals injected with type 3 reovirus that were dying of encephalitis on day 6–8 were $>5 \times 10^9$ PFU/ml, while viral titers in brains of animals injected with 3×10^5 PFU of type 1 reovirus on a comparable day were $\sim 3 \log_{10}$ lower ($\sim 5 \times 10^6$ PFU/ml). By day 15, viral titers in animals injected with type 1 had fallen to $\sim 5 \times 10^3$ PFU/ml.

Disease Patterns in Animals Injected with Hybrid Clones. Recombinant clones that segregated each of the 10 dsRNA segments were selected for animal inoculation (Table 1). Clone 65 was studied first because all the dsRNA segments coding for the outer capsid polypeptides (M2, S1, S4) were derived from type 1, while most of the genes coding for internal and nonstructural polypeptides were derived from type 3. The pattern of survival of animals inoculated with this clone was similar to the pattern in those inoculated with type 1 (Fig. 2). In addition, histological study demonstrated that some animals had ependymal cell necrosis with hydrocephalus; no animals showed evidence of neuronal necrosis. Furthermore, titers of virus from brain tissue of sacrificed animals were comparable to those seen with type 1.

Because the general pattern of virulence and cell damage induced following inoculation with clone 65 most resembled that of type 1, these early experiments suggested that one or more components of the outer capsid were playing a primary role in the neurotropism of reovirus.

Accordingly, subsequent experiments focused on recombinants that segregated genes coding for the polypeptides of the outer capsid (M2, S1, S4). Because the S1 genome segment codes for the protein ($\sigma 1$) that confers type specificity for reovirus (both in terms of neutralization and hemagglutination properties), a hybrid clone was next chosen to determine if the S1 gene was also responsible for the type-specific virulence of reovirus type 3. Hybrid clone 204 (Fig. 1 and Table 1) contains an S1 dsRNA segment from type 3 and M2 and S4 dsRNA segments from type 1; the remainder of genome segments are

Table 1. Patterns of virulence, viral titers, and histology following intracerebral inoculation of hybrid reovirus clones into newborn mice

	Origin of genome segment*												
	Outer capsid			Core			Non-structural		Uncertain		Pattern of	Viral	
Clone	M2	S1	S4	L1	L2	S2	M3	S3	L3	M1	Disease†	titers‡	Histology§
65	1	1	1	3	3	1	3	1	3	3	1	1	Normal & 1
54	1	1	3	3	1	3	3	3	1	1	1	1	Normal & 1
802	3	1	3	1	3	3	3	3	3	3	Intermediate	Intermediate	Normal & 1
80	3	1	3	1	3	1	3	3	1	3	Intermediate	Intermediate	Normal & 1
103	3	1	1	1	3	3	3	3	3	3	Intermediate	1	Normal & 1
204	1	3	1	3	1	1	1	1	1	1	3	3	3
63	1	3	3	3	1	3	1	1	3	1	3	3	3
94	3	3	1	1	3	1	3	1	3	1	3	3	3

* Numbers in the table indicate from which type of reovirus the genome segment of the hybid clone originated. The segments are grouped according to the location of their protein products in the virus.

† Pattern of disease: Type 1, infected animals generally survive without overt acute illness. Type 3, infected animals die after an incubation period of 5–10 days with occasional survivors at 3×10^2 PFU/mouse. Intermediate pattern, death usually occurs later than with type 3 and most animals survive at lower doses of viral inoculation (3×10^3 to 3×10^2 PFU/mouse).

‡ Viral titers: Infected brains have revealed three levels of peak virus titers. Type 1 = 10^5–10^6 PFU/ml. Type 3 >5×10^9 PFU/ml. Intermediate level = 10^7–10^8 PFU/ml.

§ Histology: Type 1, ependymal damage with associated hydrocephalus; neuronal cells are normal. Type 3, necrotizing encephalitis affecting neurons; ependymal cells are normal. Normal, no abnormal neuropathologic findings.

from type 1 except for L1, which is from type 3. Thus clone 204 contains eight genes from type 1 and two genes (S1 and L1) from type 3. As shown in Fig. 2, clone 204 is exceptionally virulent, analogous to, and perhaps even more virulent than, type 3. Following intracerebral inoculation, we have had no survivors with clone 204 at 3×10^2 PFU/per mouse (~20% of animals injected with 3×10^2 PFU of reovirus type 3 survive). Histologic study has shown a necrotizing encephalitis; ependymal cells have been intact in all animals studied and no animals have had hydrocephalus. Viral titers of these brains have been $>5 \times 10^9$. These experiments thus suggested that the virulence and cell tropism of type 3 is a property of the S1 gene product.

Because the S1 gene of type 3 conferred neuronal tropism and virulence into a recombinant (clone 204) containing primarily type 1 genes, it was important to determine if a reciprocal recombinant (i.e., containing a type 1 S1 gene on a predominantly type 3 background) would be avirulent and produce ependymitis and hydrocephalus. Hybrid clone 802 (Fig. 1 and Table 1) contains S1 and L1 segments from type 1, while all other genome segments are derived from type 3. As shown in Fig. 2, clone 802 is virulent at high doses (3×10^5) but is relatively avirulent at low doses (3×10^3 and 3×10^2). However, it is not as virulent as clone 204 or as the type 3 parent even at higher doses (death occurs between days 9 and 11 following inoculation with clone 802, whereas death occurs between days 5 and 7 following injection with clone 204 or type 3). Histologic study reveals that, while most animals dying after inoculation with clone 802 have normal brains, some animals show ependymal necrosis and hydrocephalus. No animals had evidence of neuronal necrosis. Viral titers from infected brains were ~5×10^8 PFU/ml, higher than those of animals injected with type 1 but lower than those from animals injected with type 3. These results provide strong evidence that a type 1 gene in the S1 position is responsible for the ependymal tropism of reovirus type 1. However, while the site of tissue destruction appears linked to the S1 gene, the fact that there is an intermediate pattern of virulence in clone 802 suggests a role for additional genes in the virulence pattern of this clone.

To further confirm the findings obtained with clones 65, 802, and 204, additional hybrid clones (54, 63, 80, 94, 103) that further segregated each of the viral genes were inoculated intracerebrally into newborn mice. The results with these clones are summarized in Table 1 and Fig. 2. In each instance, the presence of a type 3 S1 gene (clones 63 and 94) conferred a high degree of virulence associated with neuronal destruction, while a type 1 S1 gene led to ependymal damage and hydrocephalus (or a normal histologic appearance) associated with a type 1 (clone 54) or an intermediate (clones 80 and 103) pattern of death (Table 1). These results confirmed our prior conclusions concerning the primary role of the S1 gene.

DISCUSSION

The results we have obtained utilizing recombinant clones derived from crosses between reovirus type 1 and type 3 indicate that a single gene is responsible for the differing neurotropism of reovirus. The presence of an S1 gene from type 1 is responsible for the destruction of ependymal cells and the subsequent development of hydrocephalus. Ependymal damage only occurs when the S1 gene is from type 1 (clones 54, 65, 80, 103, and 802). This is most dramatically illustrated with clone 802, which has only two genome segments from type 1 (S1 and L1). The L1 segment in this hybrid is not responsible for the ependymal damage because ependymal cells are normal in animals injected with clone 94 (which has a type 1 L1 segment). The presence of an S1 gene from type 3 is responsible for the acute necrotizing encephalitis with neuronal destruction associated with type 3 virus (clones 63, 94, and 204). This is dramatically illustrated with clone 204, which has only two genome segments from type 3 (S1 and L1). The L1 segment is not responsible for the encephalitis and neuronal destruction because neuronal cells are normal following inoculation with clones 65 and 54, whose L1 segments derive from type 3. Thus, the neuronal destruction caused by reovirus type 3 is related to the S1 genome segment.

While the relationship of the S1 gene to distribution of virus in the brain ("cell tropism") appears to be absolute, the relationship of the S1 gene to death of the infected animals is somewhat more complex. Recombinants consisting of an S1 from type 3 inserted into a background of predominanty type 1 genes (such as clone 204) are extraordinarily virulent. However, the reciprocal recombinants, i.e., those in which the

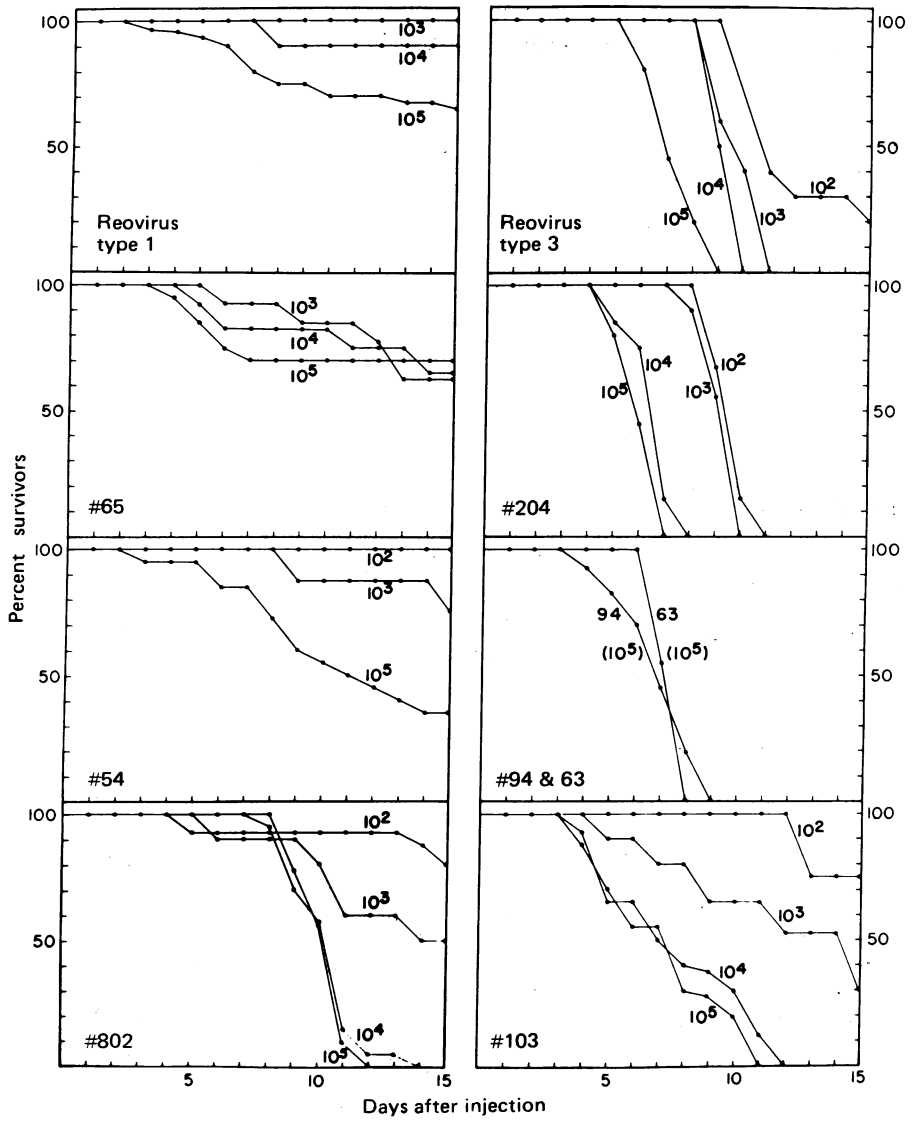

FIG. 2. Survival patterns of mice after intracerebral inoculation with parental reovirus types 1 and 3 and various recombinant clones. Newborn mice were injected intracerebrally within 24 hr of birth with various amounts of virus and studied for 15 days. Survival curves are labeled according to amount of virus injected; 10^2, 10^3, 10^4, and 10^5 represent 3×10^2, 3×10^3, 3×10^4, and 3×10^5 PFU of virus injected per mouse.

background is predominantly type 3 but which contain a type 1 S1 gene (such as 802, 103, and 54) show a reduced and delayed pattern of death that is intermediate between types 1 and 3 (802, 103) or essentially like type 1 (54). Histologically, the brains of animals that die following injection with these clones are devoid of neuronal destruction, showing either ependymal damage or no abnormalities. Thus, the mechanism of death is almost certainly different than that associated with the type 3 S1 neuronal destruction.

The differences in neurotropism and the different patterns of virulence caused by reovirus types 1 and 3 are most likely due to specific interaction of the $\sigma 1$ outer capsid protein (the protein coded for by the S1 genome segment) with receptors on the surface of either ependymal cells or neuronal cells. According to this scheme, the $\sigma 1$ protein from type 1 interacts with a cell surface receptor on ependymal cells, allowing viral penetration into the cell and subsequent ependymal cell damage. In an analogous fashion the $\sigma 1$ protein from type 3 interacts with a cell surface receptor on neuronal cells. Furthermore, the specificity of cell tropism exhibited by reovirus types 1 and 3 suggests that there is no crossreactivity between the $\sigma 1$ protein of type 1 and neuronal cells or the $\sigma 1$ protein of type 3 and ependymal cells. The fact that $\sigma 1$ is the hemagglutinin polypeptide further supports its role in receptor interactions (13).

Other investigators have suggested that cell receptors play an important role in determining viral tropism and virulence. Sabin showed that adsorption of an avirulent strain of poliomyelitis to central nervous system tissue did not occur under conditions that facilitated the adsorption of a virulent strain (14). In a similar fashion, Holland (15) has shown that susceptibility to coxsackie viruses correlates with the presence of receptors for viral adsorption on the cell surface; in several animal hosts, homogenates of susceptible organs adsorb the virion, while homogenates of insusceptible organs do not.

Mayer *et al.* (16) used a genetic approach to investigate the neurovirulence of a particular strain of influenza, A/NWS/cc-p (HON1). Recombinants between this virulent strain and a nonneurovirulent strain of influenza, A/Jap/305 (H2N2), demonstrated no link between neurovirulence and either the viral hemagglutinin or neuraminidase. Earlier genetic studies of neurovirulent strains of influenza (17–19) demonstrated a variable expression of virulence and it was generally felt that virulence was a multigenetic phenomenon.

Our studies thus differ from those with influenza because the

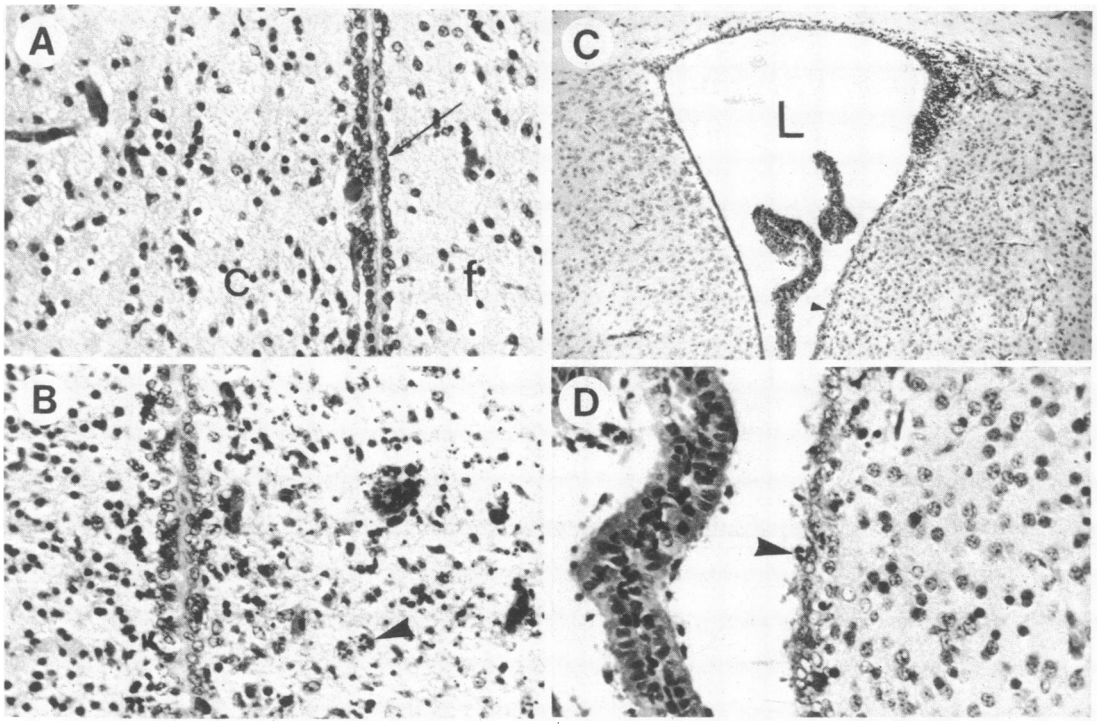

FIG. 3. Hematoxylin/eosin stains of brains from mice injected with reovirus types 1 and 3. (*A*) Normal mouse brain from 7-day-old animal: histologic appearance of the caudate/putamen (c), lateral ventricular ependyma (arrow), and septal nucleus (f). (×85.) (*B*) Brain of mouse injected with reovirus type 3 and dying on day 7: extensive neuronal necrosis within the septal nucleus (arrow) with preservation of ependymal cells. (×85.) (*C*) Brain of mouse injected with reovirus type 1 and sacrificed on day 15: greatly enlarged lateral ventricle (L) with preservation of septal nucleus and caudate/putamen. Arrow marks site of detail in *D* below. (×21.) (*D*) Detail of ventricular wall from *C* (above) showing ependymal cell necrosis represented by karyorrhexis (arrow). There is no evidence of neuronal necrosis. (×85.)

σ1 polypeptide, the reoviral hemagglutinin, is linked to both neurovirulence and cell tropism. However, our results using recombinant clones lacking a type 3 gene in the S1 position (802, 103, 54) but containing type 3 genome segments in the M2 and/or S4 positions (genes coding for the other two outer capsid proteins) suggest that multiple genes may be capable of producing virulence in an altered manner when the primary virulence gene is absent. It maybe that these "secondary virulence genes" produce their effects through control of viral replication rather than by determining specific virus/host-cell interactions.

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