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Prolonged Replication in the Mouse Central Nervous System of Reoviruses Isolated from Persistently Infected Cell Cultures

LYNDA A. MORRISON,[1]* BERNARD N. FIELDS,[1,2] AND TERENCE S. DERMODY[3,4,5]

Department of Microbiology and Molecular Genetics[1] and Shipley Institute of Medicine,[2] Harvard Medical School, Boston, Massachusetts 02115, and Departments of Pediatrics[3] and Microbiology and Immunology[4] and Elizabeth B. Lamb Center for Pediatric Research,[5] Vanderbilt Medical School, Nashville, Tennessee 37232

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We examined pathogenic characteristics of plaque-purified reoviruses isolated from persistently infected L-cell cultures (PI viruses) after intracranial inoculation into newborn mice. The PI viruses were isolated from independent cultures initiated with high-passage stocks of the wild-type (wt) strain, type 3 Dearing. The virulence of most PI viruses was equivalent to that of the wt strain. However, replication of PI viruses in the central nervous system of infected mice was prolonged to 25 (but not 50) days postinoculation. Thirty-eight percent (n = 186) of mice inoculated with the PI viruses had residual virus detectable in brain tissue 25 days after inoculation, in contrast to only 16% (n = 57) of mice inoculated with wt virus (P = 0.009). Mean residual brain titers were more than 20-fold higher in mice inoculated with PI viruses compared with wt virus (4.3×10^4 versus 2.1×10^3; P = 0.006). Tropism of PI virus within the brain resembled that of wt virus, and the distribution of PI virus antigen in the brain did not change over time. The extent of necrosis in the brains of mice harboring PI virus 25 days after inoculation was minimal, despite continued presence of high titers of infectious virus. The latter observation resembles the absence of cytopathicity seen in L-cell cultures persistently infected with reovirus. These observations suggest that the interaction of PI viruses with cells can be altered in vivo as well as in cell culture, but virus is eventually cleared from the infected animal.

Numerous viruses have the capacity to cause persistent infections in their host organisms. Establishment of persistent infection in vivo requires that the virus have a means to limit cellular injury and to evade the host immune response (reviewed in references 3 and 20). Strategies evolved by viruses to minimize damage to host cells include restricted viral gene expression (4, 9, 29) and generation of viral variants (1, 17, 27, 34). Selective cell tropism or a change in cell tropism has also been associated with the development of persistent infections (10, 26, 35). An even broader array of strategies has been utilized by viruses to escape immune detection, including reduced expression of viral proteins (4, 21, 29), modulation of major histocompatibility complex and other corecognition molecules (6, 10, 13), and infection of cells that ordinarily do not express major histocompatibility complex molecules (29). The central nervous system (CNS) is a particularly common site of viral persistence, perhaps in part because of its relative isolation from the immune system.

Serotype 3 reovirus prominently infects the CNS of newborn mice but does not establish persistent infection. It causes a highly virulent and often fatal encephalitis (16, 24, 28, 31–33), and recovery from infection is accompanied by complete clearance of infectious virus from the nervous system within approximately 2 weeks (11, 15). However, reovirus persistence in cultured murine L cells can be readily established when infection is initiated with high-passage stocks of some reovirus strains (1, 2, 5, 8). Previous studies have suggested that mutations in specific viral genes contribute to the establishment and maintenance of persistent reovirus infection in cell culture (1, 14). Insight into a possible mechanism of reovirus persistence has come from the observation that viruses isolated from persistently infected cultures (PI viruses) can grow in L cells treated with ammonium chloride (8). This finding suggests that an early, pH-sensitive step in the growth cycle of the virus is altered during persistent infections of host cells in culture.

We hypothesized that reoviruses which bear mutations associated with establishment and maintenance of persistent infections in cell culture might be altered with respect to their pathogenesis in a host organism. The virulence, tropism, and capacity to persist of 16 independently derived PI viruses were investigated after direct inoculation of virus into the CNS of newborn mice. Our findings indicate that reoviruses isolated from persistently infected L-cell cultures do possess an altered pathogenic phenotype in vivo.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted mouse L cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimum essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented to contain 5% fetal calf serum (HyClone Laboratories, Logan, Utah), 2 mM L-glutamine, 1 U of penicillin per ml, and 1 μg of streptomycin per ml (Irvine Scientific). Wild-type (wt) reovirus strain type 3 Dearing (T3D) is a laboratory stock. The PI viruses used in this study were isolated from independent persistently infected L-cell cultures initiated with high-passage stocks of T3D as described previously (8). The T3D and PI virus stocks were second-passage L-cell lysates of twice-plaque-purified viruses. Purified virion preparations of the PI

* Corresponding author.

virus, PI 7-2, were prepared from L-cell lysate stocks according to previously described techniques (12).

Mice and inoculations. Two-day-old pups born to NIH (Swiss) outbred mice, obtained from the National Cancer Institute (Frederick, Md.), were used in all experiments and were maintained in accordance with standards outlined by the institutional Animal Care and Use Committee and Public Health Service guidelines (7). The mice were administered intracranial (i.c.) inoculations with either T3D or one of the PI viruses. The virus was diluted in endotoxin-free normal saline, and a 5-μl volume was injected into the right cerebral hemisphere with a Hamilton microsyringe and a 30-gauge needle.

To determine the titer of infectious virus in brain tissue, mice were anesthetized at defined times after inoculation and euthanized by cervical dislocation. The cranium was removed, and the entire brain was placed into 1.0 to 2.0 ml of gelatin-saline and stored at −20°C. From some mice, lung, kidney, and spleen were also collected. Tissue samples were frozen and thawed twice and disrupted by sonication (Heat Systems, Farmingdale, N.Y.). Viral titer was determined in duplicate by the plaque assay technique (32).

Assessment of virulence. The 50% lethal dose (LD_{50}) after i.c. inoculation of T3D was determined by injection of serial dilutions of virus into groups of 10 mice each. The number of surviving mice in each group was noted 25 days after inoculation, and the LD_{50} was calculated by the method of Reed and Muench (25). Each of the PI viruses was inoculated at several doses in the proximity of the LD_{50} of T3D, percent survival was noted 25 days later, and an approximate LD_{50} was calculated (25). The LD_{50} of one PI virus, PI 7-2, was determined by inoculation of serial dilutions of virus as for T3D.

Statistical analyses. Data used to establish the T3D LD_{50} were fitted with a curve by second-order linear regression analysis using the Sigmaplot 3.1 program (Jandel Scientific, Sausalito, Calif.). Linear interpolation of the curve and calculation of the standard error of the mortality rate permitted a test for departure from the curve of the virulence of each PI virus. The exact test (22) was applied by using the Stat Xact 2.0 program (Cytel Software Corp.) to compare the titers of virus in brain tissue of animals surviving inoculation with T3D and those inoculated with PI viruses.

Growth of viral brain tissue isolates in L cells treated with ammonium chloride. Viral plaques were obtained from brain tissue homogenates of animals surviving 25 days after inoculation with wt and PI viruses and passaged once in L cells. First-passage stocks of these brain tissue isolates were grown in the presence and absence of ammonium chloride according to previously described techniques (8). Briefly, monolayers of L cells were infected with viral isolates in 24-well plates. After a 1-h adsorption period at 4°C, the inoculum was removed, 1.0 ml of fresh medium supplemented to contain either 0 or 10 mM ammonium chloride was added, and the plates were incubated at 37°C for 24 h. Infected monolayers were frozen and thawed three times prior to titration on fresh L-cell monolayers (32).

Tissue preparation for staining. For brain tissue collected from animals 11 to 12 days after inoculation, mice were anesthetized by intraperitoneal injection of avertin and then transcardially perfused with phosphate-buffered saline (PBS), followed by 4% formaldehyde (Fisher Scientific, Littleton, Pa.) in PBS and 10% sucrose in PBS (19). The entire brain was then removed, embedded in O.C.T. mounting medium (Miles Laboratories, Elkhart, Ind.), and stored at −70°C. For brain tissue collected from mice surviving 25 days after inoculation, mice were euthanized and brain tissue was dissected, rinsed with PBS, and divided longitudinally at the midline. One half was placed in 2.0 ml of gelatin-saline and frozen at −20°C for determination of viral titer. The other half was embedded in O.C.T. medium and frozen at −70°C for histology.

Histologic and immunohistochemical staining. Coronal sections of the entire brain were prepared from frozen tissue by using a cryostat microtome (Damon/IEC, Needham Heights, Mass.). Tissue sections were collected onto gelatin-coated slides and air dried. For histologic staining of tissues from moribund mice inoculated i.c. with $10 \times LD_{50}$ of PI 1A1, a 10-μm section was collected at 180-μm intervals and stained with hematoxylin and eosin (H&E). For histology and viral antigen staining in mice euthanized 12 days after i.c. inoculation with $1 \times LD_{50}$ of PI 1A1, consecutive sections of 12 and 30 μm were cut at 150-μm intervals and collected onto separate slides. The 12-μm sections were stained with H&E. The 30-μm sections were incubated with antireovirus antiserum (1:200 dilution of polyclonal rabbit antireovirus affinity-purified antibodies from hyperimmune serum) and then stained by the Vectastain Elite ABC immunoperoxidase rapid procedure (Vector Laboratories, Burlingame, Calif.) as previously described (19). Sections were counterstained with Giemsa (Fisher Scientific) and mounted in Permount (Sigma Chemical Co., St. Louis, Mo.). From brain tissue of mice sacrificed 25 days after i.c. inoculation with $1 \times LD_{50}$ of PI 1A1, consecutive 12- and 30-μm sections were cut at 90-μm intervals, collected onto slides, and immersed in 10% formalin fixative (Fisher Scientific) prior to staining with H&E or immunoperoxidase as described above. Control sections from uninfected mice showed no staining with the immunoperoxidase technique.

RESULTS

Virulence of PI viruses inoculated i.c. The virulence of many viruses can be attenuated by mutations that arise during high-multiplicity passage in culture. We sought to ascertain whether viruses isolated from persistently infected L-cell cultures initiated with high-passage stocks of wt reovirus strain T3D had acquired mutations that similarly decreased their virulence. We chose the i.c. route of virus inoculation because of the acute necrotizing encephalitis that is a prominent feature of T3D infection in newborn mice and to avoid any differences between wt and PI viruses in spread to the CNS from a peripheral site of inoculation. Sixteen PI viruses were compared with T3D (two isolates from each of eight independently infected cultures [8]). We first confirmed previous estimates (32) of the i.c. LD_{50} of T3D to be 9 PFU (Fig. 1). The PI viruses were then inoculated at doses approximating the LD_{50} for T3D, and the mice were monitored for survival. The lethality of 12 of the PI viruses was similar to that observed for T3D (Fig. 1). Three others were moderately attenuated compared with wt virus ($P < 0.02$); these viruses had an LD_{50} approximately threefold greater than that of wt virus. One PI virus, PI 7-2, had an LD_{50} following i.c. inoculation approximately 5.6×10^4 times greater than that of T3D. Thus, some heterogeneity exists in the virulence of PI viruses, but only 1 of 16 differed substantially from wt virus.

Duration of replication in the mouse CNS. Given that PI reoviruses can establish persistence in cell culture (1, 2, 5, 8), we investigated the capacity of these viruses to establish persistent infections in the host. Groups of mice were inoculated with $1 \times LD_{50}$ of a PI virus or wt virus by the i.c.

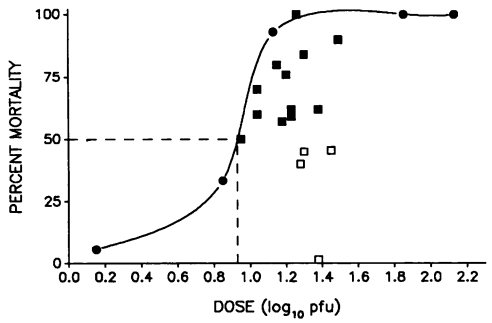

FIG. 1. Virulence of PI viruses inoculated i.c. relative to that of wt reovirus strain T3D. The percent survival of 2-day-old mice inoculated i.c. with various doses of T3D (circles) is approximated by a second-order linear regression curve (solid line). The LD_{50} for T3D is 9 PFU per mouse, as indicated by the intersection of the horizontal and vertical dashed lines. Squares indicate percent survival of groups of mice inoculated i.c. with a given dose of each of 16 PI viruses. Filled squares, PI viruses with virulence similar to that of wt virus; open squares, PI viruses whose difference in virulence from wt virus is statistically significant. Each data point represents 10 to 12 mice.

route. The viral titer in brain tissue of surviving mice was then assessed at 20, 25, and 50 days after inoculation, since the majority of animals infected with wt virus were known to have cleared the infection by these times (11, 15). The titer of virus in brain tissue of individual mice 25 days after inoculation with PI viruses or wt virus is shown in Fig. 2. Thirty-eight percent (71 of 186) of animals surviving PI virus infection had viral titer remaining, some with titers as high as 9×10^7 PFU. In contrast, only 16% (9 of 57) of animals surviving wt virus infection had detectable titer at this time ($P = 0.009$), and all of the titers were $<10^5$ PFU. The exact test (22) revealed a significant difference ($P = 0.02$) between PI viruses as a group and wt virus in titer of virus in brain tissue of all animals surviving infection. Furthermore, the mean of residual titers in PI virus-inoculated mice was

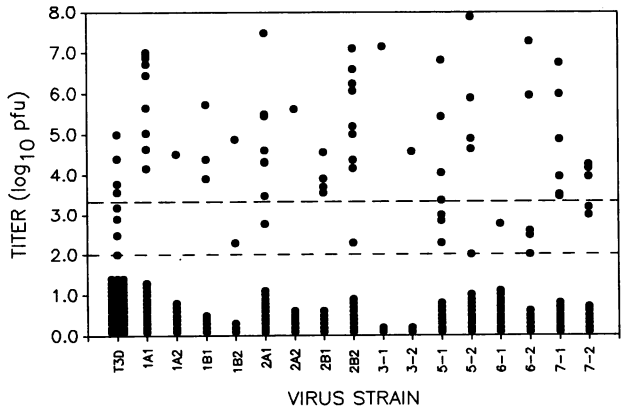

FIG. 2. Titers of virus in brain tissue of mice surviving 25 days after i.c. inoculation with wt strain T3D or the PI viruses. Each point represents the viral titer in an individual brain specimen expressed in log_{10} PFU. The upper dashed line (at 3.3 log_{10} PFU) represents the mean titer of residual virus in mice inoculated with T3D. The lower dashed line (at 2 log_{10} PFU) indicates the limit of detection of infectious virus; points below this line represent brain specimens in which no virus was detected. $n = 57$ for T3D; $n = 3$ to 20 for the PI viruses.

significantly higher than in mice inoculated with wt virus ($P = 0.006$). Thus, more than one-third of mice surviving infection with PI viruses had substantial titers of virus in brain tissue at a time when the majority of animals inoculated with wt virus had no detectable titer. Results were similar for brain tissue collected 20 days after inoculation (data not shown). However, by day 50, none of the mice inoculated with either wt or PI viruses had detectable virus remaining in the brain. In addition, no virus was detected in spleen, kidney, or lung tissue either 25 or 50 days after inoculation (data not shown). These results indicate that there is prolonged replication of PI viruses in the mouse CNS, but these viruses do not appear to establish a persistent infection.

Capacity of viruses isolated from brain tissue to grow in the presence of ammonium chloride. We previously found that PI viruses, in contrast to wt virus, exhibit normal infection and growth in L cells treated with ammonium chloride. Ammonium chloride is known to block infection by wt reoviruses at an early step in the viral growth cycle by interfering with the proteolytic processing of viral outer-capsid proteins (30). The capacity of PI viruses to grow in ammonium chloride-treated L cells suggests that they contain mutations affecting early steps in the reovirus growth cycle which appear to be important for the maintenance of persistently infected L-cell cultures (8). To determine whether PI viruses retain the capacity to grow in the presence of ammonium chloride after passage in mouse brain, we isolated viral plaques from homogenates of independent brain tissue samples obtained from animals surviving 25 days after inoculation with either T3D or one of four PI viruses, PI 1A1, PI 1A2, PI 2B1, and PI 7-1. We then compared their capacity to grow in L cells treated with 10 mM ammonium chloride. As shown in Table 1, the yields of viruses isolated from T3D-inoculated animals were significantly decreased by ammonium chloride. The yields of viruses isolated from PI virus-inoculated animals, however, were nearly equivalent at 0 and 10 mM ammonium chloride. In these experiments, the mean yields of the T3D isolates were 2.7×10^6 and 1.4×10^4 PFU/ml in untreated and ammonium chloride-treated L cells, respectively, whereas the mean yields of the PI virus isolates were 2.1×10^8 PFU/ml in untreated L cells and 9.0×10^7 PFU/ml in treated L cells. To determine the efficiency of growth of PI virus isolates relative to T3D isolates in ammonium chloride-treated L cells, we divided the yield of virus in treated L cells by that in untreated L cells (AC/L ratio) for each viral isolate (Table 1). The mean AC/L ratio was 0.0052 for the T3D isolates and 0.43 for the PI virus isolates. These results indicate that the PI virus phenotype of growth in the presence of ammonium chloride is stable during passage in mouse brain. In addition, viruses isolated from animals surviving inoculation with wt T3D do not develop the capacity to grow in ammonium chloride-treated L cells.

Distribution of PI virus-induced pathology and viral antigen in the mouse CNS. We next investigated whether PI viruses differ from wt virus in tropism within the CNS. PI virus 1A1 was selected for detailed assessment because it exhibited one of the greatest differences in mean viral titer in brain tissue compared with wt virus at 25 days after inoculation ($P = 0.002$) (Fig. 2) but did not differ from wt virus with respect to virulence (Fig. 1). We first compared the distribution of histopathologic changes in brains of mice succumbing to acute infection with PI 1A1 or T3D, reasoning that under conditions of overwhelming infection, the full extent of CNS involvement would be evident. Mice were inoculated i.c. with $10 \times LD_{50}$ of either PI 1A1 or T3D, and sections of

TABLE 1. Yields in the presence and absence of ammonium chloride of reoviruses isolated from brain tissue of animals surviving 25 days after inoculation with either T3D or PI viruses

Viral isolate[a]	Yield in[b]: 10 mM ammonium chloride	Yield in[b]: 0 mM ammonium chloride	AC/L ratio[c]
T3D-a	7.5×10^3	1.4×10^6	0.0054
T3D-b	1.8×10^4	3.9×10^6	0.0046
T3D-c	1.0×10^4	2.7×10^6	0.0037
T3D-d	2.3×10^4	2.7×10^6	0.0085
T3D-e	5.0×10^3	2.2×10^6	0.0023
T3D-f	5.0×10^3	1.3×10^6	0.0038
T3D-g	1.8×10^4	2.4×10^6	0.0075
T3D-h	2.3×10^4	5.3×10^6	0.0043
PI 1A1-a	8.0×10^7	6.7×10^7	1.2
PI 1A1-b	1.2×10^8	2.0×10^8	0.60
PI 1A1-c	2.3×10^7	1.7×10^7	1.4
PI 1A2-a	1.9×10^8	9.3×10^8	0.20
PI 2B1-a	6.2×10^7	7.2×10^7	0.86
PI 7-1-a	7.9×10^7	8.2×10^7	0.96
PI 7-1-b	6.4×10^7	1.3×10^8	0.49
PI 7-1-c	1.0×10^8	1.8×10^8	0.55

[a] Viral plaques were isolated from independent brain tissue homogenates obtained from animals surviving 25 days after inoculation with wt T3D (eight isolates), PI 1A1 (three isolates), PI 1A2 (one isolate), PI 2B1 (one isolate), and PI 7-1 (three isolates).

[b] Monolayers of L cells infected with viral isolates were incubated with or without 10 mM ammonium chloride for 24 h. Monolayers were then frozen and thawed three times and titrated on fresh L-cell monolayers. Viral yields in ammonium chloride-treated and untreated L cells are expressed as the mean of two independent experiments.

[c] Mean viral yield after 24 h of growth in ammonium chloride-treated L cells divided by that in untreated L cells for each viral isolate.

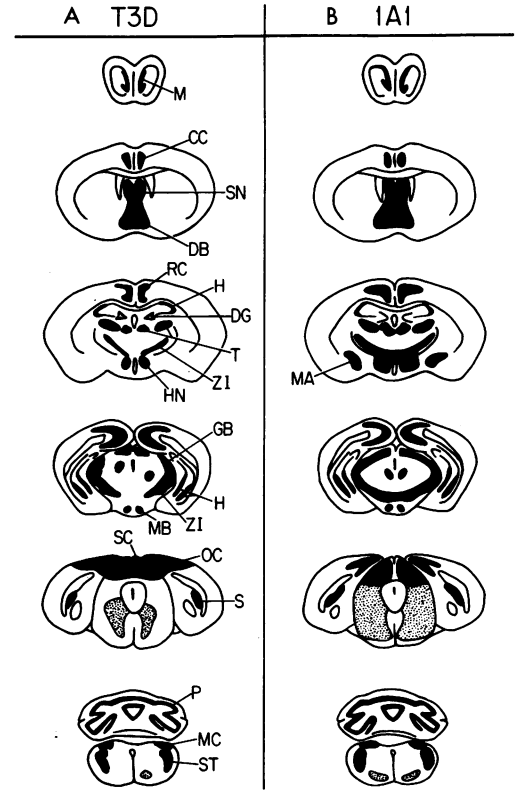

FIG. 3. Schematic representation of pathology in coronal sections of brain tissue obtained from mice 12 days after i.c. inoculation with a lethal dose of virus. (A) T3D-induced pathology, compiled from our data and the observations of Spriggs et al. (from reference 28, with permission); (B) PI 1A1-induced pathology. Solid shading indicates areas of intense tissue destruction; stippled shading indicates areas of sparse pathologic changes. CC, cingulate cortex; DB, nucleus of the ventral limb of the diagonal band; DG, dentate gyrus, pyramidal and polymorphic layers; GB, lateral geniculate body; H, hippocampus, pyramidal layer regions CA2 and CA3; HN, hypothalamic nuclei; M, mitral cell layer; MA, medial amygdaloid nucleus, anterior portion; MB, mamillary body; MC, medial cuneate nucleus; OC, occipital cortex; P, Purkinje cells; RC, retrosplenial cortex; S, subiculum; SC, superior colliculus; SN, septal nucleus, medial and lateral; ST, spinal trigeminal nucleus; T, thalamic nuclei, medial and lateral; ZI, zona inserta.

brain tissue were obtained from moribund mice 11 to 12 days later. The severity of necrosis resulting from lethal infection was equivalent for the two viruses. Brain regions exhibiting neuronal necrosis were strikingly similar in PI 1A1- and T3D-infected mice (Fig. 3). Only degenerating neurons within the dentate gyrus of wt-infected mice and in the anterior medial amygdaloid nucleus of PI 1A1-infected mice (23) were unique to each.

To determine whether the tropism of PI viruses for particular loci within the brain was altered during the course of infection, we inoculated mice i.c. with $1 \times LD_{50}$ of PI 1A1 and examined sections of brain tissue from mice that did not appear ill 12 and 25 days after inoculation. Alternating sections were stained by the immunoperoxidase technique to detect viral antigen and by H&E. The distribution of viral antigen in two mice 12 days postinoculation is shown schematically in Fig. 4A. The amount of viral antigen differed in these mice, but the distribution of antigen was similar except for a single focus of infection in the caudate putamen (Fig. 4A, mouse a). The dorsal peduncular cortex, the zona inserta, and certain thalamic nuclei appeared to be heavily infected in both mice examined, while other regions such as the cingulate and retrosplenial cortex were more moderately infected. In addition, viral antigen was confined to discrete brain nuclei and appeared to be restricted to neurons (Fig. 5A). Twenty-five days after inoculation with PI 1A1, the brain tissue of surviving mice was removed and bisected, and the viral titer of one half was determined. The complementary halves of two brains containing $>10^7$ PFU were sectioned, and alternating sections were stained by the immunoperoxidase technique and by H&E. The patterns of viral antigen distribution were identical in these two brains (Fig. 4). When the staining patterns at 12 and 25 days after inoculation were compared, it was apparent that all regions of the brain containing viral antigen 25 days after inoculation also had contained antigen at the earlier time point. These studies revealed that the distribution of viral antigen following PI 1A1 inoculation does not change with time. Infection appeared to be localized to neurons at day 25 as well as day 12 (Fig. 5B), but the more diffuse staining images obtained at day 25 precluded definitive identification of the cell type(s) infected.

Last, we compared the immunoperoxidase-stained sections with those stained by H&E that had been obtained from mice at either 12 or 25 days after inoculation with $1 \times LD_{50}$ of PI 1A1. At 12 days postinoculation, several regions of the brain, including the septal nucleus, the pyramidal layer of the hippocampus (regions CA2 and CA3), and thalamic nuclei, that were found to be heavily infected also showed significant disruption of cellular architecture. Furthermore, cytopathicity appeared limited to infected brain

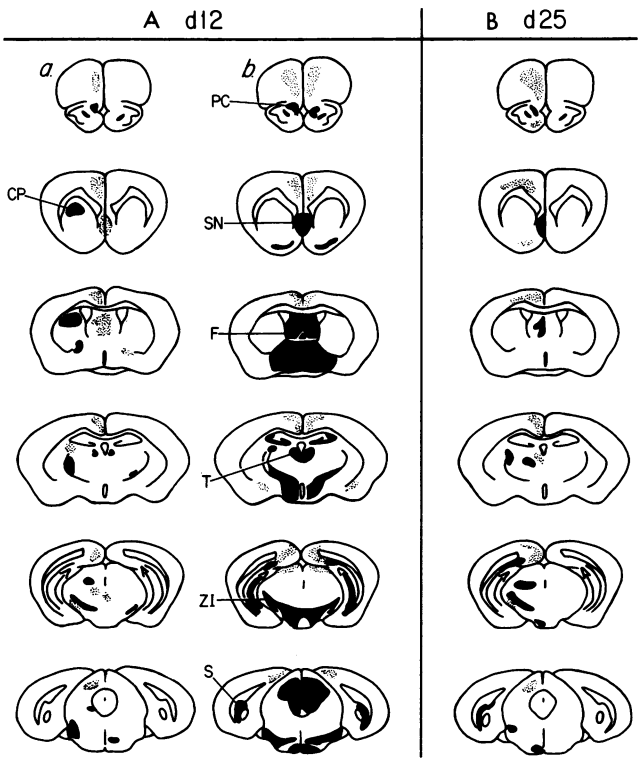

FIG. 4. Schematic representation of brain regions containing viral antigen as detected by immunoperoxidase staining in coronal sections obtained from mice inoculated i.c. with PI virus. (A) Infected brain regions in two mice (a and b) 12 days after i.c. inoculation with 1 × LD_{50} of PI 1A1; (B) infected regions observed in brain hemispheres of two mice 25 days after inoculation with 1 × LD_{50} PI 1A1. Solid shading indicates areas of intense staining; stippled shading indicates sparsely stained areas. CP, caudate putamen; F, fornix; PC, dorsal peduncular cortex; S, subiculum; SN, septal nucleus; T, thalamic nuclei; ZI, zona inserta.

regions. A striking example of involvement of discrete nuclei within the brain is shown in Fig. 5A and C. Although many pyknotic neuronal cell bodies were observed, mononuclear infiltration was limited and perivascular cuffing by inflammatory cells was minimal. In contrast, by 25 days postinoculation, regions of the brain that contained dense collections of viral antigen such as the lateral septal nucleus (Fig. 5B) appeared remarkably normal histologically (Fig. 5D), with only a few pyknotic cells and occasional evidence of neuronophagia. As was observed 12 days postinoculation, mononuclear infiltration was limited. Thus, by 25 days after inoculation, tissue damage had waned considerably despite the continued presence of high titers of virus (Fig. 2) and considerable quantities of viral antigen.

DISCUSSION

PI viruses contain mutations that play important roles in the establishment and maintenance of reovirus persistence in cell culture. In this study, we investigated whether the pathogenesis of PI viruses differs from that of wt virus in vivo. We found that a greater proportion of mice surviving infection with PI viruses than of mice surviving infection with wt virus exhibit prolonged viral presence, presumably resulting from prolonged viral replication, in the CNS following i.c. inoculation (38% versus 16%). Mean titers in brain tissue 25 days postinoculation were significantly higher in mice inoculated with the PI viruses than in those receiving T3D, the wt strain from which the PI viruses were derived. Significantly, prolonged replication of PI viruses occurred without a concomitant decrease in virulence; 12 of 16 PI viruses had an LD_{50} comparable to that of T3D after i.c. inoculation. Moreover, none of the four PI viruses with a statistically significant decrease in virulence compared with wt virus manifests prolonged replication to a greater extent than that of the other PI viruses. These findings indicate that many viral isolates obtained from persistently infected L-cell cultures also show prolonged viral replication in the mouse CNS, but this effect is not mediated through a change in virulence. This constitutes the first demonstration of an altered pathogenetic phenotype in vivo for reoviruses that establish persistent infection in cell culture.

In mice surviving a PI virus inoculum equivalent to the LD_{50}, not all brain tissue samples contained residual virus. Heterogeneity also existed among PI viruses with respect to the percentage of mice manifesting prolonged viral replication 25 days after inoculation. These results suggest that no obligate linkage exists between mutations conferring the capacity to persistently infect cells in culture and the phenotype of prolonged replication in vivo. The inconsistent occurrence of prolonged replication was not related to the dose of virus administered, because approximately the same percentage of mice with residual virus in brain tissue 25 days after inoculation was observed in survivors of doses less than the LD_{50} or as great as four times the LD_{50} (18). The major histocompatibility complex haplotype of the mice also appears not to be a factor in the observed variability of the prolonged replication phenotype. Although outbred mice were used in all of the experiments described in this report, preliminary studies using inbred mice of haplotypes $H\text{-}2^d$ and $H\text{-}2^b$ demonstrated that viral replication 20 to 30 days after inoculation is similarly prolonged in only a fraction of the mice (18). Thus, no single factor can yet explain the difference in kinetics with which individual mice clear infection with wt or PI virus.

The PI viruses used in this study were isolated from persistently infected L cells, which are of murine fibroblast origin. We found it surprising that no PI virus was detected 20 days or more after i.c. inoculation in organs rich in fibroblasts such as the spleen, liver, or lung. Serotype 3 reoviruses are known to spread beyond the CNS to peripheral organs when inoculated i.c. (18, 31). Therefore, the absence of PI virus in organs outside the CNS may indicate that PI viruses are deficient in their capacity to spread from the CNS to peripheral organs or that the CNS is unique in its permissiveness for prolonged replication of these viruses. While we cannot yet distinguish between these possibilities, the CNS has been identified as a site of persistence of several viruses (3).

We assessed whether a change in tropism was associated with prolonged replication of PI viruses, given that cellular tropism is altered in Theiler's virus persistence in the CNS (26, 35) and that a precedent exists for restricted distribution of CNS infection among variants of serotype 3 reovirus (28). We observed equally severe pathology in many brain regions of mice undergoing lethal infection with either T3D or PI 1A1. Although two regions showed evidence of unique infection by either wt or PI virus, the distribution of virus in the brains of mice 25 days after inoculation with a sublethal dose of PI 1A1 overlapped the distribution of acute wt virus infection. Second, each of the brain regions containing viral

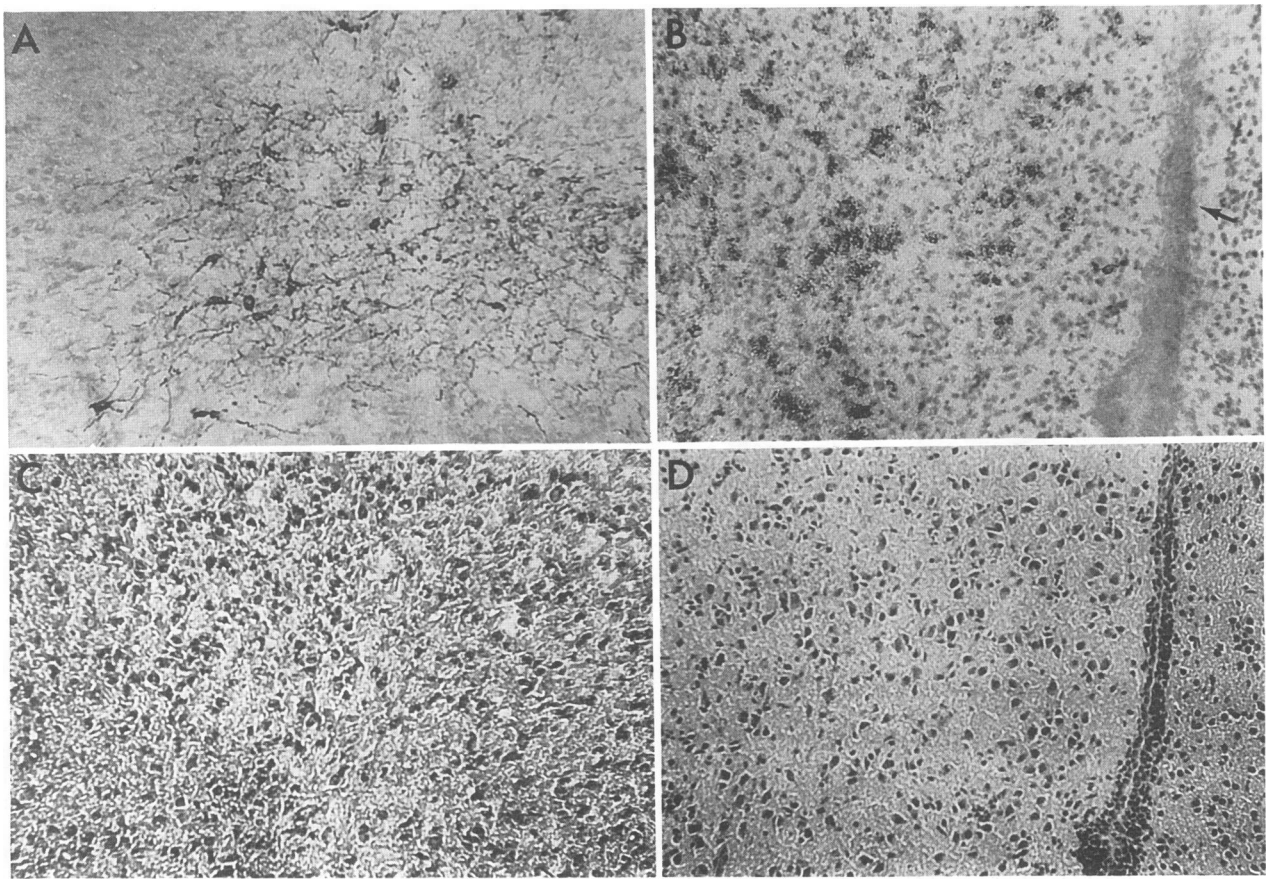

FIG. 5. Viral antigen and histopathology in coronal brain sections obtained from mice inoculated i.c. with 1 × LD_{50} of PI 1A1. (A and C) Consecutive sections through the medial thalamic nuclei 12 days after inoculation. (A) Immunoperoxidase stained; (C) H&E stained. (B and D) Consecutive sections through the septal nucleus 25 days after inoculation. The lateral ventricle is indicated by an arrow. (B) Immunoperoxidase stained; (D) H&E stained. Magnification, ×25.

antigen 25 days after inoculation with PI 1A1 also contained antigen 12 days postinoculation. Thus, no alteration or progression in the site of CNS infection that could account for the prolonged replication of PI virus was observed. Foci of heavy infection within the brain of mice 25 days postinoculation were distributed in a strikingly consistent manner, notably in regions that are interconnected via axonal projections (with the exception of the thalamic nuclei) and in part form the limbic system. Like T3D (16, 24, 33), PI 1A1 exhibited a distinct tropism for neurons 12 days after inoculation. Furthermore, cellular tropism for neurons did not appear to change during the course of PI virus infection, though evidence for this is not yet conclusive.

In order for viruses to establish persistent infections in a host organism, they must possess a means to decrease their lytic potential and evade the host immune response (3). Although PI reoviruses do not establish persistence in mice, it is likely that one (or both) of these mechanisms is involved in their prolonged replication in the mouse CNS. Extensive necrosis occurs in brain tissue of PI 1A1-infected mice 12 days after inoculation, as has been previously noted with wt virus infection (26), and this may account for the similarity in virulence of most PI viruses and wt virus. In contrast to this early time point, very little necrosis was observed in brain tissue of PI 1A1-infected mice 25 days after inoculation, despite continued high titers of infectious virus. The shift to minimal necrosis late in infection is reminiscent of the changes in cytopathicity associated with establishment and maintenance of persistent reovirus infection in cell culture. L-cell cultures infected with high-passage stocks of T3D undergo a crisis characterized by extensive cytopathic effect as persistent infection is established. As the cultures are maintained, production of infectious virus continues with minimal cytopathicity (1, 2, 8). Whether prolonged replication in vivo is associated with mutations in the viral genes implicated in establishment and maintenance of reovirus persistence in cell culture (1, 14) remains to be determined. The observation of diminished necrosis at late times after infection in vivo may reflect decreased lytic potential of the PI viruses or may indicate that particular neuronal subpopulations infected by PI virus in vivo are less susceptible to lysis.

During maintenance of persistently infected L-cell cultures, reoviruses evolve mutations that affect early steps in the viral growth cycle (8). PI viruses isolated from these cultures have developed the capacity to grow in the presence of ammonium chloride (8), a weak base that blocks proteolytic processing of infecting virions in an endocytic compartment during viral entry into cells (30). It is possible that this property alters the virus-cell interaction in the context of the host organism as well and contributes to prolonged PI virus replication in the mouse CNS. Notably, the phenotype of

growth in the presence of ammonium chloride remained stable after passage in brain tissue of newborn mice. In addition, none of the viruses isolated from brain tissue of T3D-inoculated animals developed the capacity to grow in ammonium chloride-treated L cells, suggesting that the duration of wt virus replication in the mouse CNS was insufficient to allow selection of the ammonium chloride-resistant phenotype.

A change in PI virus that alters host immune recognition represents a second potential means of producing prolonged replication in the mouse CNS. A striking inflammatory response accompanied by perivascular cuffing has been observed in the CNS in association with wt virus infection (31). In contrast, the minimal perivascular cuffing and mononuclear cell infiltration observed at both 12 and 25 days after PI 1A1 inoculation suggests that the immune response is blunted in PI virus-infected mice. Immune recognition is not completely abrogated, however, since viral clearance eventually occurs. Whether persistence could be established by PI viruses in an immunocompromised host remains a central issue.

Because viruses isolated from persistently infected L-cell cultures often manifest prolonged replication in the CNS but not true persistence, PI reoviruses appear to have only partially accomplished the goals of nonlytic replication and escape from immune-mediated clearance that are required for establishment of persistent infection in vivo. Prolonged replication rather than true persistence is intriguing and may represent an intermediate step in the evolution of the persistent state in vivo. The observed differences between persistence in cell culture and prolonged replication in the context of the host may permit dissection of the contributions of virus and host to the development of persistence in an infected animal.

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