Penetration of the Nervous Systems of Suckling Mice by Mammalian Reoviruses

ANNE FLAMAND,^{1†*} JEAN-PIERRE GAGNER,¹ LYNDA A. MORRISON,¹ AND BERNARD N. FIELDS^{1,2}

Department of Microbiology and Molecular Genetics, Harvard Medical School,¹ and Department of Medicine, Brigham and Women's Hospital, and The Shipley Institute of Medicine,² Boston, Massachusetts 02115

Received 18 June 1990/Accepted 20 September 1990

Penetration of the nervous systems of suckling mice by prototype strains of the three mammalian reovirus serotypes was studied after footpad inoculation of a dose (10^7 PFU) representing 3.5×10^3 50% lethal doses (LD_{50}) for reovirus type 3 Dearing and less than 1 LD_{50} for reoviruses type 1 Lang and type 2 Jones. Type 3 Dearing entered both motor and sensory neurons; infected neurons were clearly detectable by immunohistochemical staining 19 h after inoculation. By day 2, a second cycle of infection had occurred, and by day 4, several hundred motor and sensory neurons and interneurons were infected. By this time, infection also involved large areas of the brain stem and brain. There was evidence of both retrograde and anterograde movement of viral antigen within axons and dendrites. Unexpectedly, reovirus type 1 Lang followed neuronal pathways as well as being disseminated in the bloodstream. Reovirus type 2 Jones also entered neurons. While the number of motor neurons and interneurons infected with type 1 Lang or type 2 Jones remained limited within the first 4 days after inoculation, infection of sensory neurons increased with time and reached a level by day 4 comparable to that observed after infection with type 3 Dearing. Viral antigen was also found in the brain stem and brain, but this infection was limited. These three strains multiplied in nonneuronal tissues. Connective tissue in the footpad was massively infected by all three strains 19 h after inoculation. By this time, foci of infection were also present in muscle and skin. Viral antigen was repeatedly observed in the endothelium of blood vessels and in the meninges after infection with type 1 Lang. The titer of type 1 Lang increased in the blood with time, which was not observed after infection with strains of the other two serotypes. In this study, we found that prototype strains of the three reovirus serotypes exhibited different degrees of neurotropism, all being capable of entering neurons. Transmission of the infection occurred through synapses rather than from cell body to cell body. Thus reovirus, like herpesvirus and rabies virus, is a good marker for the identification of neuronal pathways, although its capacity to grow in neurons, unlike that of herpesvirus and rabies virus, is restricted to newborn animals.

Mammalian reovirus strains share many structural and biological properties, among which is an icosahedral capsid containing 10 double-stranded RNA genome segments (reviewed in reference 16). Three serotypes have been identified and are commonly represented by the prototypic strains type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D) (16). Strains of the three serotypes manifest different patterns of cellular injury *in vivo* (reviewed in reference 19). T3D spreads via nerves when injected into newborn mice (20), causing lethal necrotizing encephalitis (19). T1L infection produces nonlethal endependymitis (9, 14), although occasional infected neurons have been observed (11, 14). T2J has been reported to cause nonlethal encephalitis in suckling mice (26), but the neuropathology caused by this strain has not been well characterized.

In mixed infection with two strains of reovirus, gene segment reassortment can occur, resulting in progeny that contain gene segments from both parents (15). Comparison of reassortants generated between T1L and T3D has demonstrated that the localization of neural injury (encephalitis) is a property of the T3D S1 gene (28, 29), while the magnitude of viral growth in the central nervous system (CNS) is related to the M2 gene (7). Using reassortant viruses, the capacity of reovirus to spread via nerves from

the footpad to the brain has been associated with the T3D S1 gene (20). The S1 gene encodes the outer capsid protein $\sigma 1$, which is the viral hemagglutinin and cell attachment protein (13, 27).

Although T3D has been shown to spread to the CNS via nerves (20), the details of how serotype 3 reoviruses enter the nervous system are not known. In this report, we studied the penetration and propagation of prototype strains of the three reovirus serotypes in the CNS after inoculation into the hindlimb footpad. The innervation of the footpad permits the virus easy access to termini of both motor and sensory neurons, whose cell bodies are located in the lumbar segments of the spinal cord and corresponding sensory ganglia, respectively. We examined serial sections of the spinal cord and sensory ganglia for the presence of viral antigen to determine whether virus enters by the motor and/or sensory route(s). A novel finding was the capacity of the T1L and T2J strains as well as the T3D strain to enter neurons rapidly at the site of inoculation, indicating that these prototype strains of the three reovirus serotypes are each capable of penetrating the CNS through nerves.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted mouse L cells were propagated as previously described (6). Cell concentration was adjusted daily to 4.5×10^5 cells per ml by dilution of the culture with Joklik modified Eagle minimum essential medium (Irvine Scientific, Santa Ana, Calif.) supplemented

* Corresponding author.

† Present address: Laboratoire de Génétique des Virus, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cédex, France.

with 5% fetal calf serum (HyClone Laboratories, Logan, Utah), 2 mM glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin (Irving Scientific) per ml. Reoviruses T1L, T2J, and T3D were laboratory stocks. Viruses were doubly plaque purified, passaged two (T1L, T3D) or three (T2J) times on L-cell monolayers, and purified as described previously (6) with the following modifications. L cells were suspended at 10^7 cells per ml in low-endotoxin Eagle minimum essential medium with Earle's salts (Whittaker Bioproducts, Walkersville, Md.) containing 5% fetal calf serum and 2 mM glutamine and were infected with passage 2 or 3 virus stocks at a multiplicity of infection of 5 to 10. Cells were incubated at 34°C for 1 h and then diluted with completed Eagle medium to 5×10^5 cells per ml and maintained in suspension culture at 34°C until the viable cell count fell by 25 to 50% as determined by trypan blue dye exclusion. All subsequent steps were done in plasticware with reagents prepared in low-endotoxin water (Whittaker Bioproducts). After cells were harvested and virus was extracted as previously described (6), the aqueous upper phase from the last extraction was layered on top of a self-forming 1.25- to 1.45-g/ml cesium chloride gradient in 10 mM Tris chloride (pH 7.4) and centrifuged at 4°C for 20 h at $1.2 \times 10^5 \times g$. The viral band was collected with a syringe by side puncture and dialyzed twice against 1,000 volumes of a 10 mM Tris chloride (pH 7.4)–15 mM MgCl₂–0.15 M NaCl buffer and a third time against Dulbecco's phosphate-buffered saline (Whittaker Bioproducts) supplemented with 15 mM MgCl₂. Virus stocks were stable at 4°C and free of aerobic bacteria as determined by brain heart infusion broth cultures (Scott Laboratories, Fiskeville, R.I.). The 50% lethality of these three strains after intracerebral inoculation in newborn mice was verified as 10 PFU for T3D and $>10^7$ PFU for T1L and T2J.

Inoculation and dissection of mice. One- to two-day-old mice (CR:NIH/Swiss) born of pregnant mothers obtained from the National Cancer Institute (Frederick, Md.) were inoculated in the right hind limb footpad with a Hamilton syringe and a 30-gauge needle. At this age and by this route of inoculation, mice were not yet resistant to 10^7 PFU of reovirus T3D; this resistance begins between 4 and 6 days of age (6a). By the intracranial route, mice have not yet acquired resistance to infection by either T1L or T3D by 6 days of age (17). A 5-µl sample of approximately 2×10^9 PFU of virus per ml in Dulbecco's phosphate-buffered saline was injected into each animal. In one experiment, the virus was inoculated in the right thigh. Dissection of the spinal cord and ganglia was usually performed on nonfixed tissues. Animals were sacrificed by decapitation at specific time intervals. Samples (50 µl) of blood were removed and diluted in 1 ml of gelatin saline (0.3% gelatin, 0.14 M NaCl, 0.27 mM CaCl₂, 0.84 mM MgCl₂, 19.4 mM H₃BO₃, 0.13 mM Na₂B₄O₇) for virus titration when needed. The lumbar segments of the spinal cord with the proximal segment of sciatic nerve and five attached sensory ganglia (one below and one above the three sciatic ganglia) were dissected on ice with the aid of a stereomicroscope. Spinal cords were placed vertically in a cupule of wrapping plastic filled with O.C.T. compound embedding medium (Tissue-Tek; Miles Inc., Elkhart, Ind.), frozen in a mixture of dry ice and methanol, and stored at -70°C. Spinal cords from two animals were usually mounted together and their respective positions in the block were recorded. The right and left hind limb footpads of each animal (and in one case the right and left thigh muscles) were also removed and included in one block.

To obtain optimum frozen sections of brain and brain stem tissues, preliminary fixation of mice with formaldehyde was necessary. Animals were anesthetized with Avertin (1.25% solution of a 2:1 [wt/wt] mix of tribromoethanol in amylene hydrate) and kept over an ice bath. They were perfused through the left ventricle with successive 5-ml volumes of borate-buffered saline (BBS; 0.17 M H₃BO₃, 0.12 M NaCl, pH 8.0), BBS containing 3.7% formaldehyde, and BBS containing 10% sucrose. After dissection, the brain and brain stem were incubated overnight at 4°C in BBS containing 10% sucrose and then mounted in embedding solution. The spinal cord and ganglia were dissected and immediately frozen as described above.

Serial 30-µm transverse sections of the frozen material were made with a cryostat microtome at -12°C. Sections were recovered on gelatin-coated slides, and loss of material in no case exceeded 5%. A total of 150 to 200 sections per cord or brain and 100 to 120 sections per brain stem were collected.

Viral antigen detection. Sections made from formaldehyde-fixed tissue were treated for 20 min at room temperature with 1% Triton X-100 in enzyme-linked immunosorbent assay (ELISA) buffer (0.15 M NaCl, 0.05 M Tris chloride [pH 7.4], 1 mM disodium EDTA, 0.05% Tween 20, 0.1% bovine serum albumin). Sections made from nonfixed tissues were incubated for 20 min at 4°C with a 1:1 (vol/vol) mixture of acetone and methanol, followed by two washes with ELISA buffer. Alternatively, they were immersed for 20 min at room temperature in BBS containing 3.7% formaldehyde, followed by two washes with ELISA buffer containing 1% Triton X-100. Endogenous peroxidase activity was quenched by incubating the sections for 3 min in 0.3% H₂O₂. A modification of the VectaStain rapid procedure (VectaStain Elite ABC kit; Vector Laboratories, Burlingame, Calif.) was used. Three successive incubations were performed, each of 20 min at 37°C: first, with a 1/200 dilution of a hyperimmune anti-reovirus T3D rabbit antiserum that cross-reacted with reoviruses T1L and T2J (5a; 25); second, with a 1/200 dilution of a goat anti-rabbit immunoglobulin G biotinylated antibody; and third, with a mixture of avidin and biotinylated peroxidase (VectaStain, premixed 20 min before use). All dilutions and washes were made in ELISA buffer; dilution of primary antiserum was supplemented with 1.5% normal goat serum (VectaStain).

The fixation of peroxidase was revealed by immersing the sections for 2 to 3 min in a fresh solution of 0.1% diaminobenzidine tetrahydrochloride in 0.1 M Tris chloride (pH 7.4) mixed with an equal volume of 0.02% H₂O₂. Sections were rinsed under running water and counterstained for 2 to 3 min with filtered 2.5% Giemsa stain (Fisher, Fair Lawn, N.J.) in distilled water. They were then rinsed with running water, drained, air dried, and mounted in Permount (Fisher).

Virus titration. Blood samples were frozen (-70°C) and thawed (22°C) three times and homogenized for 15 s with a W-225R sonicator and a microtip probe set at 40% maximum output (Heat Systems-Ultrasonics, Plainview, N.Y.). All specimens were serially diluted in gelatin saline and assayed in duplicate for the presence of infectious virus on confluent L-cell monolayers in six-well dishes (Costar, Cambridge, Mass.) as described previously (25). Viral titers were reported as mean PFU per milliliter of blood, with corresponding standard errors corrected for the small sample size (5).

Determination of LD₅₀. A 5-µl sample of each serial dilution of virus was inoculated into the hindlimb footpad of litters of 10 2-day-old mice. Mice were monitored for survival for 4 weeks. The 50% lethal dose (LD₅₀) for reovirus

T3D was calculated by the method of Aubert (1). For reoviruses T1L and T2J, the mortality reached 45 and 60%, respectively, at the highest dose of virus (8.0×10^7 and 9.8×10^8 PFU per mouse, respectively). Therefore, an LD_{50} could not be determined for these two viruses. Animals for which T2J infection was lethal died within 24 h of inoculation, with occasional evidence of liver necrosis and gut infarct.

RESULTS

Infection of nonneuronal tissues. Mice (1 to 2 days old) were inoculated with 7×10^6 to 4×10^7 PFU of reovirus T1L, T2J, or T3D in the right hindlimb footpad or thigh. A dose of 10^7 PFU represents 3.5×10^3 LD_{50} for T3D and a sublethal dose of virus for T1L and T2J ($LD_{50} > 5 \times 10^7$ PFU). Cryostat sections were prepared from either the right and left footpads or thigh muscle 19, 45, 72, and 96 h after inoculation. Every fifth section was incubated with antireovirus antiserum, and the infected tissues were examined by indirect immunostaining techniques. The three strains multiplied extensively in connective tissue. Viral antigen was present in skin (Fig. 1A) and muscle (Fig. 1C and D). Three days after inoculation in the thigh and four days after footpad inoculation, considerable extension of the infection in muscle was noted with T3D (Fig. 1C) but not with T1L (Fig. 1D), consistent with the observations of K. L. Tyler (17a, 21). In addition, T1L antigen was repeatedly found in the endothelium of both arteries and veins (Fig. 1B). Viral antigen in endothelial cells was not seen after T3D infection despite extensive investigation, nor after T2J infection in a more limited number of animals. By day 4, small foci of T1L-infected cells were present in the opposite limb (data not shown).

Presence of virus in the blood. Virus titers in the blood were measured at selected times between 3 h and 4 days after inoculation. Small amounts of all three virus strains, representing between 10^{-3} and 10^{-4} of the inoculum, were initially present in the blood. There was little, if any, subsequent increase in the titer of either T2J or T3D (Fig. 2). In contrast, a 100-fold increase in the T1L titer was observed after 4 days. This increase could be related to the multiplication of the virus in the endothelium of blood vessels. Alternatively, virus could be disseminated by the blood to other tissues (e.g., reticuloendothelial system), where ensuing replication could augment the titer of virus in the blood through secondary viremia.

Penetration and propagation in the nervous system. Upon inoculation in the right hindlimb footpad, virus has access to sensory nerve termini located in the skin or muscle and to motor endings in the muscle. The corresponding axons form part of the right sciatic nerve, with sensory fibers originating at cell bodies located in the right spinal ganglia and motor fibers originating in the right side of the spinal cord (Fig. 3). Spinal ganglia contain several types of sensory neurons differing in size (Fig. 4E and F and 5E), but no attempt was made to determine the distribution of viral antigen among these categories of neurons. The gray matter of the spinal cord contains motor neurons and interneurons, of which only motor neurons extend to the periphery. Motor neurons can be easily differentiated from interneurons by their large size, intense staining with Giemsa stain, and grouping at the lower periphery of the ventral horn (Fig. 4A and B and 5A to C). Spinal ganglia and the cord also contain nonneuronal cells such as glial cells which can be distinguished from motor or sensory neurons by their smaller size.

Serial sections of the lumbar part of the cord and right spinal ganglia were observed for the presence of virus at 14, 19, and 45 h and 4 days after inoculation in the right hindlimb footpad. Since the portion of spinal cord and ganglia innervating the right hindlimb was examined totally, it was possible to determine precisely the first appearance of viral antigen in neuronal cell bodies.

(i) **T3D.** A few infected motor neurons were detectable at 14 h postinfection (Fig. 4A); infection of both motor and sensory neurons was clearly visible at 19 h (Fig. 4B and E). Eight animals were examined, and in four the quality of the sections was sufficient to allow the quantification of infected neurons (Fig. 6). An average of 80 motor and 17 sensory neurons were infected in each animal. The intensity of immunoperoxidase reaction product in both categories of neurons was usually comparable. At these early times, no immunostaining was visible in axons and dendrites of infected neurons, as has been the case with rabies virus (4).

After 45 h, the number of infected motor and sensory neurons increased two- and fourfold, respectively (Fig. 6). A few interneurons on the right side of the spinal cord were also positive, suggesting that spread to adjoining nerve termini and a second cycle of replication had occurred by this time. At 4 days after infection, several hundred motor and sensory neurons were infected on the right side of the cord as well as more than 500 interneurons (Fig. 4C, D, and F). On the left side of the cord, many motor neurons and interneurons were also infected, but the left sensory ganglia were not. Viral antigen was present in both axons and dendrites of interneurons (Fig. 4C) and was detected in the dorsal and ventral roots of the spinal cord. Virus antigen-positive axons and dendrites were always associated with infected cell bodies, either on the same or on an adjacent tissue section. This fact, together with the observation that such processes were not observed at early times (14 and 19 h), argues against a nonspecific labeling of injured processes. Examination of the thoracic spinal cord revealed the presence of several hundred infected interneurons, mostly on the right side (data not shown). The meninges and the choroid plexus did not show any evidence of viral infection with T3D.

To determine the extent of infection of T1L and T2J relative to T3D, the numbers of infected motor or sensory neurons of all animals examined at each time point have been averaged in the following comparisons.

(ii) **T1L.** Reovirus T1L exhibited some ability to infect neurons (Fig. 5B to E and 6). Infection by T1L was more impaired in motor neurons than in sensory neurons. No infected motor neurons were found at 19 h in the lumbar spinal cord of six animals, and invasion of sensory neurons represented 6% of what was found in T3D-infected mice.

At 45 h after infection, a few motor as well as sensory neurons contained viral antigen (3 and 9% of what was observed for T3D, respectively), but the motor neurons could have been infected from sensory neurons. It is therefore not certain whether T1L is capable of penetrating motor neurons at the periphery. In view of the finding of Tyler et al. (20) that the titer of T1L was equivalent in the inferior and superior portions of the spinal cord 1 to 7 days after inoculation in the hindlimb, we studied the presence of the virus in motor neurons and interneurons of the thoracic and cervical portions of the spinal cord. In the two infected animals examined, 10 and 11 positive motor neurons were found on the right side of the lumbar section of the cord. In one animal, the rest of the cord was entirely negative, and in the other, only two interneurons were found infected. There-

FIG. 1. Infection of nonneuronal tissues by mammalian reoviruses. (A and C) T3D. (A) Foci of infected cells (arrow) in the epidermis (S, skin) 19 h after inoculation in the footpad. Connective tissue (C) on the right side of the picture shows uninfected but purple-stained cells that are also present in uninoculated animals (data not shown). (C) Heavy infection of the muscles (M) by virus (arrow) 3 days after T3D inoculation in the thigh. (B and D) T1L, 3 days after inoculation in the right thigh. (B) Presence of virus (arrow) in the endothelium of blood vessels (V) in the left thigh. (D) Small foci of infected cells (arrow) in the right thigh muscles. Magnification: A, $\times 80$; B, C, and D, $\times 28$.

fore, neural spread of T1L would not explain the previous findings of equal virus titers in inferior and superior portions of the spinal cord. It is likely that virus titer present in extracts of the cord as measured in the experiments of Tyler et al. (20) was derived not only from neurons but also from the blood, endothelium of blood vessels, and meninges that

are all collected during the dissection. In the T1L-infected animals, the virus observed in neurons would not be likely to contribute significantly to the titer, while for T3D, which was not found in endothelium or meninges, neurons would contain most of the virus.

By day 4, the number of infected motor or sensory

FIG. 2. Virus in the blood after footpad inoculation with T1L, T2J, or T3D. Two-day-old mice were inoculated in the right footpad with 5 μ l of purified virus and sacrificed at 3, 24, 48, 72, or 96 h after inoculation. Blood samples of 50 μ l were collected, and the titer of virus in the blood and in the inocula (arrows) was determined by plaque assay. Plotted points represented the mean titer of virus from four animals with corresponding standard errors corrected for the small sample size. Symbols: $\circ$, T1L; Δ , T2J; $\square$, T3D.

neurons increased markedly and, in the sensory system, almost reached the level observed in T3D-inoculated animals (Fig. 6). Animals infected with T1L differed strikingly from those infected with T3D in that only a few interneurons were found infected and were located on the right side of the cord. Positive neurons were also found in the brain stem and to a lesser extent in the brain (data not shown). Many foci of infection were observed in the meninges (Fig. 5F), but the choroid plexus showed very little viral antigen (data not

shown). Ependymal cells were not infected by 4 days after inoculation.

(iii) **T2J.** Little is known about the way in which T2J infects the nervous system; foci of neuronal infection have been observed in thalamic nuclei and cerebellar peduncles of mice inoculated orally (26). In our study of animals inoculated in the footpad with T2J, few motor neurons were positive at 19 h (1% of what was observed with T3D). This increased to 2% after 45 h (Fig. 6, left panel). Infected sensory neurons were more numerous than motor neurons at 19 and 45 h (19 and 28% of that found for T3D, respectively), reaching a level higher than that observed in T1L-infected animals (Fig. 6, right panel). At 4 days, involvement of motor neurons remained limited; sensory neurons were more massively invaded, but only on the injected side (Fig. 6).

DISCUSSION

We demonstrated that prototype strains of the three serotypes of mammalian reoviruses are capable of infecting motor and sensory neurons after footpad inoculation in neonatal mice. Thus, the capacity to enter neurons is not serotype specific. The entry of virus into nerves and subsequent neural spread of the infection was consistently lower for T1L and T2J than for T3D. Prior studies with reovirus T1L after peripheral or intracerebral inoculation have shown that the primary CNS pathology of T1L infection is ependymal cell injury (9, 14). Reovirus T1L has been occasionally observed in neurons (11, 14), although overt encephalitis has not been reported. After footpad inoculation, each strain first appeared in motor and/or sensory neurons in the lumbar spinal cord and/or sensory ganglia on the side of inoculation. This indicates that the spread of virus follows neuronal pathways to the CNS as previously described for T3D (20), instead of being spread through the bloodstream. In addition to replication in neurons, intense viral replication occurred at the site of inoculation. Both T3D and T1L replicated in skeletal muscle, as had been previously observed (11, 17a, 19, 21), but extension of the infection in muscle apparently occurred more efficiently with T3D.

T3D was found simultaneously in motor and sensory neurons 19 h after inoculation (although a few infected motor neurons were detected 5 h earlier). This implies that virus

FIG. 3. Schematic representation of potential sensory and motor neuronal pathways traveled by reovirus from hindlimb muscles to the lumbar spinal cord. Motor innervation of the muscle spindles as well as most connections of sensory and motor neurons have been omitted for clarity. Solid lines represent sensory and motor nerve fibers. Dashed lines represent interneurons. Categories of neurons which could be primarily or secondarily infected are indicated by filled cell bodies.

FIG. 4. Presence of T3D-infected neurons (arrows) in the spinal cord (A, B, C, and D) and sensory ganglia (E and F) as revealed by immunoperoxidase staining. Infected motor neurons 14 h (A) and 19 h (B) after inoculation in the right footpad. Motor neurons are large and are located in the right ventral horn of the cord. Infected neurons 4 days after inoculation: (C) interneurons in the left ventral horn; (D) motor neurons and interneurons. Uninfected motor neurons are present at the bottom of panel C and contrast with heavily infected motor neurons in the right ventral horn shown in panel D. Infected sensory neurons were found in spinal ganglia 19 h (E) and 4 days (F) after inoculation. Magnification: A and B, $\times 80$; C, $\times 164$; D, $\times 23$; E, $\times 44$; F, $\times 96$.

enters the nervous system by both motor and sensory routes, as has been described for rabies virus (4), another neurotropic virus capable of invading the CNS after forelimb and hindlimb inoculation. Does the virus directly penetrate nerve termini? The appearance of viral antigen in cell bodies

of the spinal cord and ganglia depends on the time required for axonal transport and for each cycle of replication. Reovirus is transported within axons innervating the footpad by fast axonal transport (20), which operates at a speed of 2 to 10 mm/h (12). In newborn mice, this would yield a

FIG. 5. Presence of TIL (arrows) in neurons and meninges (Mn) 4 days after inoculation in the right footpad. (A) Uninfected control; spinal cord (SC) and two sensory ganglia (SG). Motor neurons are heavily contrasted and are situated laterally in the ventral horns. (B) Spinal cord with infected motor neurons on the right side. (C) Infected motor neurons (enlargement of a section similar to panel B). (D) Infected axons exiting the ventral motor route (MR). (E) Infected sensory neurons in spinal ganglia. (F) Foci of infection in meninges (Mn). Cryostat sections were from either unfixed (A, E, and F) or Formalin-fixed (B, C, and D) tissues as described in Materials and Methods. Magnification: A and B, $\times 15$; C, $\times 80$; D, $\times 222$; E, $\times 45$; F, $\times 90$.

transport time of approximately 1.5 to 7 h from the footpad to the spinal cord after virus enters nerve termini in the footpad. Reovirus progeny are estimated to be produced as early as 4 to 6 h (2) or 8 to 15 h (3) after inoculation in intestinal tissue in vivo and 12 h in L cells in vitro (5a). Therefore, we currently cannot distinguish whether reovirus directly enters nerve terminals or requires a cycle of replication prior to entry.

Between 19 and 45 h postinfection, there was a marked

increase in the number of infected motor and sensory neurons on the right side of the lumbar segments of the spinal cord and ganglia. This could be due either to reinfection of motor neurons by sensory neurons (or vice versa) via reflex arcs or to additional entry of virus into the CNS from the primary site of replication. Delayed infection could contribute to lethality provided it occurs before age-related acquisition of resistance to infection. Another possibility that cannot be excluded is that the virus spreads through the

FIG. 6. Number of infected motor (left panel) and sensory (right panel) neurons after footpad inoculation with T1L, T2J, and T3D. The total number of motor and sensory neurons per inoculated animal was counted on serial cryostat sections of the lumbar segments of the spinal cord and related ganglia after immunostaining. Symbols: $\circ$, T1L; Δ , T2J; $\square$, T3D. The dashed segment of the line for T3D represents a conservative extrapolation of that portion of the curve because in two of three T3D-infected animals examined 4 days after inoculation, antigen-positive motor neurons were too numerous to count. The value shown for the third animal thus represents the lowest estimate of the actual number of infected motor neurons in these animals.

bloodstream to other parts of the body and then may reach the CNS from these secondary foci of infection. This is unlikely, however, because motor and sensory neurons on the left side of lumbar cord segments (contralateral to infection) are not infected by 45 h and are infected on both sides of thoracic and cervical segments only occasionally by day 4.

The lethality of neurovirulent viruses after a hindlimb, forelimb, or intracranial inoculation appears to be due for the most part to the infection and death of neurons. Bearing in mind differences in experimental design, reovirus T3D and the CVS strain of rabies virus show a similar efficiency in neuronal penetration when the number of neurons infected by these viruses is compared soon after inoculation. For reovirus T3D, newborn mice inoculated with 3.5×10^3 LD₅₀ (1 LD₅₀ = 2.8×10^3 PFU) in 5 μ l have an average of 97 motor and sensory neurons infected by 19 h, hence 1 infected neuron per 36 LD₅₀. For rabies virus, adult mice inoculated in the forelimb with 400 LD₅₀ (1 LD₅₀ = 5.0×10^4 PFU, calculated according to Aubert [1]) in 10 μ l have an average of 14 neurons infected by 18 h (Table 1 in reference 4), hence 1 infected neuron per 29 LD₅₀.

Virus was certainly transported from the periphery to cell bodies, i.e., retrogradely, although viral antigen could not be visualized in the peripheral axons. Viral antigen could be seen in spinal axons of sensory neurons 45 h after inoculation (data not shown), suggesting that anterograde spread occurs after sufficient viral replication in cell bodies. Evidence of both retrograde and anterograde transport of reovirus is also provided by the spread of infectious T3D to retinal ganglion cells after intracranial inoculation (18) and to the spinal cord after footpad inoculation (20). Therefore, as previously observed in vivo for rabies virus (4) and herpes

simplex virus (10, 22, 23), reoviruses appear to be transported in an anterograde as well as retrograde direction in the axon.

Although T3D penetrates the nervous system by both motor and sensory routes, there were always more motor than sensory neurons infected until at least day 4. In the spinal cord, the majority of the motor neurons innervating the footpad muscles appears infected at 4 days, while the involvement of sensory neurons in sciatic ganglia remains more limited. In footpad sections, the invasion of muscles was massive, yet only dispersed foci of infection could be seen in the skin where most sensory endings are located. The amount of replication at the primary site of infection may thus directly determine the spread of virus into the CNS. Also notable is the observation that cell bodies of infected neurons in sensory ganglia were well separated and thus probably became infected from their termini rather than by contact of cell bodies.

T2J and, unexpectedly, T1L exhibited a capacity to enter peripheral neurons. However, subsequent infection of the CNS appeared to be restricted because animals did not develop encephalitis and did survive the inoculation. The capacity of T1L and T2J to infect sensory neurons within the first 45 h was significant, representing 9 and 28%, respectively, of what was observed for T3D, but the capacity to infect motor neurons was 3% or less of that observed for T3D. Nevertheless, 4 days after infection many motor neurons became infected, indicating that motor neurons were indeed permissive for strains of all three serotypes. It is not known whether T1L penetration at the periphery was impaired, or whether viral synthesis was slower, in most motor neurons compared with sensory neurons. Notably, a reduced efficiency of T1L multiplication in muscle was observed. If indeed this reflects a requirement for local entry at a particular site, decreased replication in muscle could contribute to the reduced number of infected motor versus sensory neurons observed, a conclusion similar to that reached for T3D.

T1L also multiplied in the endothelium of blood vessels, which is consistent with the finding that the titer of T1L in the blood steadily increased during the 4 days after inoculation, in contrast to the lack of significant increases in titer observed after T2J and T3D infection. Foci of infection were also frequently detected in the meninges of the cord and brain in T1L-inoculated animals. Our findings are consistent with previous reports of T1L infection in the endothelium of blood vessels (11, 24) and in the meninges (11), as well as the rise of its titer in the blood (8, 11). We saw no evidence of T1L infection in ependymal cells over the 4 days after inoculation and suspect that the T1L-induced ependymitis (9, 14) occurs at a later stage of infection. Examination of brain pathology after peripheral inoculation of T1L will be required to establish this point. Characterization of the neurons of the cord and brain which are invaded by strains of each serotype is in progress.

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REFERENCES

1. Aubert, M. F. A. 1980. Méthodes simples d'interprétation statistique des essais biologiques à réponses individuelles du type "tout ou rien." *Bull. Ec. Natl. Supér. Agron. Nancy* 22:51-60.
2. Bass, D. M., J. S. Trier, R. Dambraskas, and J. L. Wolf. 1988. Reovirus type I infection of small intestinal epithelium in suckling mice and its effect on M cells. *Lab. Invest.* 55:226-235.
3. Bodkin, D. K., M. L. Nibert, and B. N. Fields. 1989. Proteolytic digestion of reovirus in the intestinal lumens of neonatal mice. *J. Virol.* 63:4676-4681.
4. Coulon, P., C. Derbin, P. Kucera, F. Lafay, C. Prehaud, and A. Flamand. 1989. Invasion of the peripheral nervous systems of adult mice by the CVS strain of rabies and its avirulent derivative Av01. *J. Virol.* 63:3550-3554.
5. Dixon, W. J., and F. J. Massey, Jr. 1969. Introduction to statistical analysis, 3rd ed., p. 136, 487. McGraw-Hill Book Co., New York.
- 5a. Flamand, A. Unpublished observation.
6. Furlong, D. B., M. L. Nibert, and B. N. Fields. 1988. Sigma 1 protein of mammalian reoviruses extends from the surfaces of viral particles. *J. Virol.* 62:246-256.
- 6a. Gagner, J.-P. Unpublished observation.
7. Hrady, D. B., D. H. Rubin, and B. N. Fields. 1982. Molecular basis of reovirus neurovirulence: role of the M2 gene in avirulence. *Proc. Natl. Acad. Sci. USA* 79:1298-1302.
8. Jenson, A. B., E. R. Rabin, D. C. Bentinck, and F. Rapp. 1966. Reovirus viremia in newborn mice. An electron microscopic, immunofluorescent and virus assay study. *Am. J. Pathol.* 49:1171-1183.
9. Kilham, L., and G. Margolis. 1969. Hydrocephalus in hamsters, ferrets, rats, and mice following inoculations with reovirus type I. *Virologic studies. Lab. Invest.* 21:183-188.
10. Kristensson, K., I. Nennesmo, L. Persson, and E. Lycke. 1982. Neuron to neuron transmission of herpes simplex virus. Transport of virus from skin to brainstem nuclei. *J. Neurol. Sci.* 54:149-156.
11. Kundin, W. D., C. Liu, and J. Gigstad. 1966. Reovirus infection in suckling mice: immunofluorescent and infectivity studies. *J. Immunol.* 97:393-401.
12. Kuypers, H. G. J. M., and G. Ugolini. 1990. Viruses as trans-neuronal tracers. *Trends Neurosci.* 13:71-75.
13. Lee, P. W. K., E. C. Hayes, and W. K. Joklik. 1981. Protein $\sigma 1$ is the reovirus cell attachment protein. *Virology* 108:156-163.
14. Margolis, G., and L. Kilham. 1969. Hydrocephalus in hamsters, ferrets, rats, and mice following inoculations with reovirus type I. *Pathologic studies. Lab. Invest.* 21:189-198.
15. Ramig, R. F., T. A. Mustoe, A. H. Sharpe, and B. N. Fields. 1978. A genetic map of reovirus. II. Assignment of the double-stranded RNA-negative mutant groups C, D, and E genome segments. *Virology* 85:531-544.
16. Schiff, L. A., and B. N. Fields. 1990. Reoviruses and their replication, p. 1275-1306. In B. N. Fields, D. M. Knipe, et al. (ed.), *Virology*, 2nd ed. Raven Press, New York.
17. Tardieu, M., M. L. Powers, and H. L. Weiner. 1983. Age dependent susceptibility to reovirus type 3 encephalitis: role of viral and host factors. *Ann. Neurol.* 13:602-607.
- 17a. Tyler, K. L. Unpublished observation.
18. Tyler, K. L., R. T. Bronson, K. B. Byers, and B. N. Fields. 1985. Molecular basis of viral neurotropism: experimental reovirus infection. *Neurology* 35:88-92.
19. Tyler, K. L., and B. N. Fields. 1990. Reoviruses, p. 1307-1328. In B. N. Fields, D. M. Knipe, et al. (ed.), *Virology*, 2nd ed. Raven Press, New York.
20. Tyler, K. L., D. A. McPhee, and B. N. Fields. 1986. Distinct pathways of viral spread in the host determined by reovirus S1 gene segment. *Science* 233:770-774.
21. Tyler, K. L., H. W. Virgin IV, R. Bassel-Duby, and B. N. Fields. 1989. Antibody inhibits defined stages in the pathogenesis of reovirus serotype 3 infection of the central nervous system. *J. Exp. Med.* 170:887-900.
22. Ugolini, G., H. G. J. M. Kuypers, and A. Simmons. 1987. Retrograde transneuronal transfer of herpes simplex type 1 (HSV1) from motoneurons. *Brain Res.* 422:242-256.
23. Ugolini, G., H. G. J. M. Kuypers, and P. L. Strick. 1989. Transneuronal transfer of herpes virus from peripheral nerves to cortex and brainstem. *Science* 243:89-91.
24. Verdin, E. M., S. P. Lynn, B. N. Fields, and E. Maratos-Flier. 1988. Uptake of reovirus serotype 1 by the lungs from the bloodstream is mediated by the viral hemagglutinin. *J. Virol.* 62:545-551.
25. Virgin, H. W., IV, R. Bassel-Duby, B. N. Fields, and K. L. Tyler. 1988. Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). *J. Virol.* 62:4594-4604.
26. Walters, M. N., P. J. Leak, R. A. Joske, N. F. Stanley, and D. H. Perret. 1965. Murine infection with reovirus. III. Pathology of infection with types I and II. *Br. J. Exp. Pathol.* 46:200-212.
27. Weiner, H. L., K. A. Ault, and B. N. Fields. 1980. Interaction of reovirus with cell surface receptors. I. Murine and human lymphocytes have a receptor for the hemagglutinin of reovirus type 3. *J. Immunol.* 124:2143-2148.
28. Weiner, H. L., D. Drayna, D. R. Averill, Jr., and B. N. Fields. 1977. Molecular basis of reovirus virulence: role of the S1 gene. *Proc. Natl. Acad. Sci. USA* 74:5744-5748.
29. Weiner, H. L., M. L. Powers, and B. N. Fields. 1980. Absolute linkage of virulence and central nervous system cell tropism of reovirus to viral hemagglutinin. *J. Infect. Dis.* 141:609-616.