Type 3 Reovirus Neuroinvasion after Intramuscular Inoculation: Direct Invasion of Nerve Terminals and Age-Dependent Pathogenesis

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INTRODUCTION

Neonatal mice are vulnerable to reovirus neuroinvasion from peripheral sites of infection. After hindlimb injection, type 3 reovirus travels via the sciatic nerve to replicate in spinal cord motor neurons before spread to the brain and development of lethal encephalitis. Here we provide ultrastructural evidence for direct reovirus invasion of unmyelinated neonatal motor nerve terminals within 2 h and replication in spinal cord motor neurons within 14 h after hindlimb injection of 1-day-old mice. In adult mice, resistance to reovirus lethality after intracranial (IC) injection correlates with the restriction of virus growth in cortical neurons. We found that neuroinvasion also is age dependent after intramuscular injection. Virus lethality and CNS infection decreased sharply during the first postnatal week, while lethality after IC injection continued for 2 additional weeks. Mice inoculated at 7 days of age with high virus doses suffered paralysis of the injected limb, but significant brain infection was not lethal. These results suggest that reovirus invasion of the neonatal CNS is restricted by several progressive age-dependent mechanisms.

There are striking differences between the adult motor unit and that of the newborn (Slater, 1982; Salpeter, 1987; Betz, 1987). In the adult, each spinal cord motor neuron may innervate hundreds of muscle fibers via its branched axon. Each of these muscle fibers is innervated by only one branch at a single motor endplate, the axon branch is myelinated, and the nerve–muscle junction is tightly "capped" by Schwann cells. By contrast, in the newborn each muscle fiber receives axon terminals from several motor neurons, the preterminal motor axons are unmyelinated, and Schwann cells only loosely surround the nerve–muscle junction. The excess of immature nerve terminals in the newborn allows for the postnatal refinement of neural circuitry. Within 2 weeks after birth, the number of nerve terminals has been reduced by at least 50% as axon branches withdraw, leaving singly innervated neuromuscular junctions, the remaining axon terminal has been myelinated and the nerve–muscle junction capped by Schwann cells (Colman and Lichtman, 1993; Salpeter, 1987). Thus, during the first days after birth, motor neurons may be especially vulnerable to reovirus invasion.

In this study, we wished to test the hypothesis that after hindlimb injection type 3 reovirus directly invades neonatal sciatic nerve terminals and to determine whether one or more steps in reovirus neuroinvasion is age dependent. We used reovirus type 3 clone 9 (C9), because 50% lethal doses by the IC and IM routes of injection are similar in 1-day-old mice, thus providing a
good basis for comparison of subsequent age-related and/or route-dependent differences.

RESULTS

Virus invasion of nerve terminals

To determine whether neonatal sciatic nerve terminals are exposed to injected virus particles, we sacrificed 1-day-old mice at 90 min after hindlimb injection with C9 virus and examined thin sections of skeletal muscle by electron microscopy. Near the injection site, virus particles appeared to be excluded from contact with larger nerve branches by the perineural sheath of fibroblasts (Fig. 1B) and many were engulfed by resident phagocytes (data not shown). As expected, the preterminal axon (within 35 μm of the synapse) was unmyelinated (Fig. 1A). Here, we occasionally found virus particles closely apposed to the axon membrane and the Schwann cell that surrounded it (Fig. 1D). We also observed reovirus particles among the axon profiles at multiply innervated nerve–muscle junctions, but not at the active zone where the basal lamina occupied the interstitial space (Fig. 1C). Although muscle fiber profiles were abundant in many sections, viral particles were seen only occasionally in association with muscle membrane.

At this early time point, we also found evidence of virus uptake into the axonal cytoplasm. Virus particles were seen in endosome-like vesicles near the active zone (Fig. 2A) and in preterminal axons (Figs. 2B and 2C). In addition to virus particles, these endosomes sometimes contained small spherical profiles resembling synaptic vesicles (Fig. 2C). Single virus particles were seen also in endosomes in muscle fibers and Schwann cells. Thus, by 90 min after injection, reovirus particles had invaded both sciatic nerve terminals and muscle fibers near the injection site.

Because our micrographs suggested that the uptake of reovirus particles might occur along the unmyelinated preterminal motor axon as well as near the active zone in newborn mice in vivo, we examined the uptake of C9 virus in primary cultures of mouse ventral spinal cord neurons. In cultures fixed at 90 min after infection, we found endosomes containing viral particles and spherical vesicles similar to those seen in 1-day-old mice (Fig. 3A). In addition, we observed a virus particle apparently in the process of endocytosis in a vesicle with the electron-dense coat characteristic of a coated pit (Fig. 3B). Because the vesicle membrane was still continuous with the cell membrane, the axonal site of uptake was clear. These results suggest that reovirus uptake may occur in unmyelinated motor axons, such as those found in primary cell cultures and near the sciatic nerve terminals of neonatal mice.

Kinetics of factory formation

If virus uptake into sciatic nerve terminals results in productive infection, viral replication would be expected to begin as infectious particles are transported to the neural soma located in the ventral spinal cord. To determine whether virus replication occurs in the neonatal spinal cord at a time consistent with direct infection of sciatic nerve terminals, we prepared vibratome sections from spinal cords collected at 15 and 19 h after C9 hindlimb injection. Infected neurons were identified by immunohistochemical detection of viral antigen (Fig. 4A) and subsequently examined by electron microscopy for evidence of the distinctive cytoplasmic reovirus assembly structures, viral “factories,” characteristic of reovirus replication (Raine and Fields, 1973; Fig. 4B). As a standard for comparison of the morphology and kinetics of factory formation, we prepared a time course of micrographs of C9 infection of the mouse fibroblast cell line used to grow and titer reovirus. In these cells, small replication factories could be detected by 6 h after infection, and factories rapidly increased in number and size over the next 6 h (Fig. 4C and Table 1).

Factory morphology in motor neurons in infected spinal cords was similar to that seen in cell culture, but the kinetics of factory formation were somewhat slower (Fig. 4C and Table 1). By 15 h after hindlimb injection, perinuclear replication factories in spinal cord motor neurons contained up to 57 viral particles, and by 19 h after injection they contained more than 200 particles. By comparison, our examination of sections made from infected muscle tissue collected at 12 h after IM inoculation revealed factories similar to those found in spinal cord neurons at 15 h. Thus, replication in hindlimb muscle fibers was about 3 h ahead of that in spinal cord neurons, suggesting that replication in spinal cord motor neurons was well underway before progeny virus was formed in muscle fibers. Given the distance that virus must travel to reach the neural soma (about 14 mm), this timing was consistent with a model in which virus invasions of nerve terminal and muscle fiber are parallel events, with the kinetics of viral replication reflecting the different distances virus must travel before reaching the cytoplasmic machinery necessary for viral replication.

Age dependence of neuroinvasion

To determine whether neuroinvasion of clone 9 virus was dependent on the age of the mouse, we first measured 50% lethal doses (LD50) after inoculation by the IC and IM routes in mice from 1 to 14 days of age (Fig. 5). The LD50 after IM injection increased rapidly until postnatal day 7, when even the highest virus dose (102 pfu) killed no mice. By contrast, the LD50 by the IC route increased more gradually over 3 weeks. Thus, mice were vulnerable to IC injection well after lethality had ceased by the IM route. The route-specific difference in LD50...
FIG. 1. Virus association with sciatic nerve terminals at 90 min after IM injection. A. Multiple motor axons innervate a muscle fiber (lower left) at the neuromuscular junction. The preterminal axon remains unmyelinated until synapse elimination has removed all but one motor terminal (Salpeter, 1987). In this preterminal region, virus particles (arrows, upper left) can be found (see enlarged view in D). B. Virus particles accumulate at the membrane of perineural cells (upper right) which surround a bundle of unmyelinated axons (lower left). C. Virus particles (arrowheads) are seen between two axon terminals. The basal lamina (*) lies parallel to muscle and nerve membranes but is not seen between axon terminal membranes. D. Virus particles (arrowheads) were seen between two axons, an axon and a Schwann cell (S), and in the interstitial space near collagen fibers. Note the cluster of synaptic vesicles (v) in an axon near the center of the micrograph.
increased from less than 10^1 pfu in newborn mice to greater than 10^5 pfu in 7-day-old mice, suggesting that one or more early steps in reovirus invasion was age dependent.

We next compared C9 viral growth in muscle and its spread to spinal cord and brain in mice of increasing age (Fig. 6). Tissue was collected on alternate days after low-dose (10^3 pfu) IM injection (2–12 mice per time point, total 111 mice). Virus grew well in muscle in mice up to 2 weeks of age, but titers were greatly reduced in 3-week-old mice. Virus rapidly spread to spinal cord and brain in 1- and 3-day-old mice, but titers increased more slowly in 7-day-old mice and were virtually absent in 2-week-old mice. These results suggested that both virus infection of skeletal muscle and invasion of the CNS were age dependent. However, the gradual decrease in virus growth at the injection site did not appear to account for the more rapid restriction of virus spread to spinal cord and brain.

Although hindlimb injection was not lethal in 7-day-old mice at any dose (Fig. 5), mice injected with high doses of virus developed flaccid paralysis of the inoculated limb by 7–9 days after injection. In these mice, spinal cord virus titers reached 10^6 pfu within 7 days (Fig. 7). Brain titers (10^7.7 pfu) were similar to those produced by lethal IC injection of mice of the same age, yet no mice died, suggesting a route-specific response to virus infection.

**DISCUSSION**

The pathogenesis of viral invasion of the CNS involves many distinct steps, including replication at the primary site of infection, entry and spread within the CNS, growth in neural tissue, host immune response, and tissue injury (Tyler and Fields, 1996). Each of these steps may be...
influenced by viral and/or host factors. Reovirus infection of neonatal mice has provided a useful animal model for studying the pathogenesis of virus-induced CNS disease. Using neurotropic type 3 clone 9 reovirus, we provide evidence for direct virus invasion of immature motor nerve terminals and demonstrate age dependence at several steps in neuroinvasion after hindlimb injection of newborn mice.

Direct entry into nerve terminals

After hindlimb injection, type 3 reovirus rapidly invades the spinal cord and brain of newborn mice. Virus first appears in the inferior spinal cord, where viral antigen has been detected in motor neurons within 14 h after injection (Flamand et al., 1991). However, immunohistochemical detection of viral antigen cannot distinguish

FIG. 4. Kinetics of virus replication in spinal cord neurons. A. By 16 h after IM injection of 1-day-old mice, a small number of C9 virus-infected ventral spinal cord neurons can be detected by immunohistochemistry (note the neuron with a lightly stained cytoplasm near the center of micrograph). B. Ultrastructural examination of these stained cells (A) allows visualization of individual viral particles in replication factories in neural cytoplasm. Image shown is from a thin section stained with uranyl acetate and lead citrate. C. Comparison of the number of virus particles in the largest viral factories found by ultrastructural examination of ventral spinal cord neurons at 15 and 19 h after IM injection and in L-cell cultures at 1, 6, 8, 10, and 12 h after infection. *Number of virus particles in the largest factory found in thin sections of infected muscle fibers 12 h after hindlimb injection.

influenced by viral and/or host factors. Reovirus infection of neonatal mice has provided a useful animal model for studying the pathogenesis of virus-induced CNS disease. Using neurotropic type 3 clone 9 reovirus, we provide evidence for direct virus invasion of immature motor nerve terminals and demonstrate age dependence at several steps in neuroinvasion after hindlimb injection of newborn mice.
among endocytosed viral particles, early viral protein synthesis, and newly assembled viral particles. Thus it could not be determined whether virus had replicated in the neural soma after direct entry at the nerve terminal or had been transported to the neural cell body after replication in muscle fibers.

By ultrastructural examination of infected neonatal tissue in this study, we were able to trace early events in C9 neuroinvasion following IM injection. Rather than accumulation of injected virus particles at the muscle membrane or preferential uptake in the postsynaptic muscle cytoplasm, we observed single reovirus particles in endosomes in muscle fibers as well as in the unmyelinated preterminal motor axons characteristic of immature nerve–muscle junctions. Thus, reovirus infection of muscle and nerve appear to be parallel events, occurring within 90 min after hindlimb inoculation.

In mature neurons, endocytosis occurs in the preterminal axonal membrane at the margins of the active zone, where synaptic vesicle membrane is recycled after transmitter release (Brodin et al., 2000; Heuser and Reese, 1973). However, in developing neurons synaptic vesicle recycling also occurs along axons and may play a trophic role during the maturation of innervation (Matteoli et al., 1992). Although nerve–muscle junctions are formed before birth, the motor nerve terminals mature during postnatal remodeling as polynervous innervation is eliminated and the preterminal axonal membrane is myelinated. In our study, this immature region of the motor terminal appears to be vulnerable to reovirus invasion. Whether axon terminals withdrawing from multiply innervated endplates, terminals moving from multiply innervated endplates to form synapses on adjacent secondary muscle fibers (Duxon et al., 1986), or the single terminal which remains to innervate the mature neuromuscular junction is the primary target for reovirus invasion is a subject for further study.

Using sciatic nerve section and colchicine treatment, Tyler et al. (1986) demonstrated that reovirus travels to the spinal cord via the microtubule-associated system of fast axonal transport. Because reovirus particles can associate with microtubules when replicating in infected cells (Raine and Fields, 1973; Parker et al., 2002) and with isolated microtubule preparations in vitro (Babiss et al., 1979), it has been suggested that virus particles may be transported directly on microtubules. However, at least

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**TABLE 1**

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**FIG. 5.** C9 LD<sub>50</sub> in mice of increasing ages. Each point represents the 50% lethal dose (LD<sub>50</sub>) for groups of mice injected at the indicated age with various doses of C9 virus by the intracranial (IC) or intramuscular (IM) route. *No lethality, even at highest dose.
early infection of neonatal motor nerve and primary neural cell cultures, C9 virus appears to travel in endosomes (Figs. 2B and 2C; Fig. 3A). Viral entry into the endosome–lysosome degradation pathway activates viral replication as lysosomal proteases uncoat the virus particle during transport to the perinuclear cytoplasm where progeny viral particles are formed (Nibert et al., 1996). Retrograde fast axonal transport moves at 2–3 mm/h in vivo, somewhat more slowly than in cell culture (Nathanson and Tyler, 1997). Thus, the viral factories we observe in motor neurons in the ventral spinal cord at 15 h after IM injection may reflect 4–7 h of axonal transport. Consistent with ultrastructural evidence for direct virus penetration of immature motor nerve terminals, factory formation within 15 h postinoculation suggests that prior replication in muscle fibers is not required for reovirus invasion of neonatal peripheral nerve. However, as with rabies virus, replication at the injection site may amplify the viral inoculum and contribute to continuing spread to the CNS (Charlton et al., 1997; Flamand et al., 1991).

Direct virus invasion of peripheral nerve and spread by fast axonal transport appear to be common features of neurotropic viruses (reviewed in Tyler and Gonzalez-Scarano, 1997). Rabies virus, like reovirus, enters neural pathways after hindlimb injection. However, invasion is species and strain specific but not age dependent (Watson et al., 1981; Coulon et al., 1989; Shankar et al., 1991). The proposed site of entry is the nerve–muscle junction, where reovirus virus particles accumulate by binding to acetylcholine receptors in the postsynaptic muscle membrane (Lentz et al., 1982; Lewis et al., 2000). Such binding may position viral particles for subsequent entry into the presynaptic nerve terminal by binding to neural acetylcholine or P75 receptors (Tuffereau et al., 1998). In our study, reovirus particles did not appear to accumulate at the postsynaptic muscle membrane but were taken up into the preterminal axon. Reovirus is known to bind to sialic acid residues (Tyler, 1998) and to junction adhesion molecule (Barton et al., 2001); however, the nature of the reovirus receptor on neonatal nerve terminals remains to be defined.

### Age dependence of pathogenesis

It has been shown previously that T3D lethality after IC injection is restricted within 10 days after birth and correlates with decreased capacity of cranial neurons to support virus replication (Tardieu et al., 1983). We found a somewhat longer susceptibility of neonatal mice to IC injection with C9 virus. This may reflect differences in

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**FIG. 6.** Virus spread in mice of increasing ages after IM injection. Curves represent the titer of C9 reovirus in muscle (A), spinal cord (B), or brain (C) in groups of mice injected with $10^3$ pfu of C9 at the indicated ages and sacrificed at days 1, 3, 5, or 7 postinjection. Points represent mean titers ± SE.

**FIG. 7.** Virus growth in 7-day-old mice. The curves represent virus titers in muscle spinal cord or brain after intramuscular injection of 7-day-old mice with C9 ($10^3$ pfu). Points represent mean titers ± SE. *C9 brain titer 7 days after IC injection ($10^3$ pfu/mouse) of 7-day-old mice.
viral replication induces IL-1 in inoculated mice. Recent studies demonstrate that C9 and cytokine induction will be measured in IC- and IM-7-day-old mouse, and anti-reovirus antibody response matures. Immune responses may also be altered in the and/or induction of apoptosis occur in the brain as mice will determine whether differences in antigen distribution additional route-specific mechanisms. Future studies will determine whether differences in antigen distribution and/or induction of apoptosis occur in the brain as mice mature. Immune responses may also be altered in the 7-day-old mouse, and anti-reovirus antibody response and cytokine induction will be measured in IC- and IM-inoculated mice. Recent studies demonstrate that C9 viral replication induces IL-1α in the brains of neonatal but not adult mice, suggesting that the neonatal immune response may contribute to reovirus lethality (Derrien and Fields, 1999). However, induction is not dependent on the route of inoculation, and the response of 7-day-old mice has not yet been determined. Lawson and Perry (1995) report that the neonatal inflammatory response undergoes an abrupt but transitory increase in 1-week-old mice, exceeding that of both the newborn and the adult. Thus, in 7-day-old mice, intracranial injection of virus may elicit a protective response not produced by the nontraumatic introduction of virus by neural transport.

Induction of limb paralysis by high-dose IM injection of 7-day-old mice suggests that the mechanism that limits the lethality of brain infection in mice of this age does not protect spinal cord neurons from injury. Paralysis has not been a feature of recent reports of T3D and C9 infection. Early reports of reovirus type 3 hepatoencephalomyelitis virus described paralysis as an early symptom of lethal infection in neonates (Papadimitriou, 1966). C9-induced paralysis without lethality in maturing mice resembles poliovirus infection, in which adolescents are susceptible to nonlethal limb paralysis (Jubelt et al., 1980).

Taken together, our results suggest that reovirus invasion of the developing CNS after hindlimb injection is limited by several progressive age-dependent mecha-
lated with each of three dilutions, and the titer was determined by standard plaque assay (Tyler et al., 1985).

Detection of viral antigens
Reovirus antigens were detected in paraformaldehyde-fixed vertebrate sections by the immunoperoxidase method using a rabbit polyclonal anti-T3D antiserum (Tyler et al., 1989) and a Vectastain Elite ABC reagent kit (Vector Laboratories).

Electron microscopy
Virus antigen-positive areas of spinal cord or muscle vertebrate sections were postfixed in uranyl acetate, embedded in epon–araldite, sectioned, and stained with lead citrate and uranyl acetate. Grids were examined using Hitachi or Joel transmission electron microscopes. L-cell or neural cell cultures were infected with C9 at a m.o.i. of 100 pfu per cell and incubated at 37°C for 1–14 h. Cells were fixed in 4% paraformaldehyde/0.05% glutaraldehyde and processed for electron microscopy.

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REFERENCES
Tyler, K. L., and Gonzalez-Scarano, F. (1997). Viral diseases of the