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Direct spread of reovirus from the intestinal lumen to the central nervous system through vagal autonomic nerve fibers

(viral pathogenesis/myenteric plexus/Peyer's patch/poliovirus/encephalitis)

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ABSTRACT **A crucial event in the pathogenesis of systemic enteric virus infections is entry of virus into the nervous system. Whether enteric virus spreads from the intestinal tract to the central nervous system through nerves or through the bloodstream was examined using a serotype 3 reovirus strain. After peroral inoculation of newborn mice with reovirus, serial histologic sections of small intestine, brain, and spinal cord were prepared and stained by immunoperoxidase to detect viral antigen. Three days after inoculation, viral antigen was observed in mononuclear cells of ileal Peyer's patches and in neurons of the adjacent myenteric plexus. Infection first appeared in the central nervous system 1–2 days later in neurons of the dorsal motor nucleus of the vagus nerve. Endothelial cells, meninges, choroid plexus, hypothalamus, and area postrema were not infected, indicating neural rather than blood-borne spread from the intestine. Staining of neurons in the dorsal motor nucleus of the vagus nerve depended on the route of virus inoculation and was independent of the amount of virus in the bloodstream. These results demonstrate that an enteric virus entering a host from the intestinal lumen can spread to the central nervous system through nerve fibers innervating the intestine.**

A fundamental unresolved issue in systemic infections with viruses entering a host through the gastrointestinal tract is the route by which virus penetrates the central nervous system (CNS) to cause encephalitis (1–4). Virus could potentially enter the nervous system by direct spread from infected plexuses of nerve fibers in the wall of the alimentary tract (5) or from nerve endings in secondarily infected muscle and visceral organs (6, 7). Alternatively, virus could gain entry to the bloodstream and then penetrate the nervous system through the meninges or through blood vessel endothelium in sites where the blood–brain barrier may be more permeable (8–10): the choroid plexus, hypothalamus, and area postrema. For poliovirus, the most well-studied neurotropic enteric virus of humans, the preponderance of opinion currently favors blood-borne spread to the CNS once viremia has commenced (2, 3, 11, 12), but spread by direct peripheral nerve penetration has not been disproven.

The mammalian reoviruses are a group of enteric viruses that have been useful in deciphering the genetic basis of viral pathogenesis (13). Reoviruses enter a host from the intestinal lumen through M cells overlying ileal Peyer's patches (14) and undergo primary replication in the intestine (15, 16). Three serotypes have been identified (13). Serotype 3 strains are neuronotropic, and infection of newborn mice ultimately results in a lethal encephalitis (17). That serotype 3 reoviruses have the potential to spread through nerves has been demonstrated after footpad inoculation of strains Dearing (T3D; refs. 18 and 19) and clone 9 (T3C9; K. Tyler and H. Virgin, personal communication). After peroral inoculation, T3C9, like many other serotype 3 strains, also rapidly enters the bloodstream and produces viremia (20). Infection with strain T3C9 thus represents a useful nonprimate system to investigate whether enteric viruses penetrate the CNS from the intestinal tract by hematogenous or neural routes.

The small intestine is innervated primarily by sympathetic and parasympathetic fibers of the autonomic nervous system and by sensory nerve fibers. Neuronal cell bodies of each of these fiber types have an anatomically distinct location that can be observed in histologic sections: parasympathetic in the dorsal motor nucleus of the vagus nerve (DMNV), sympathetic in the intermediolateral cell column of the thoracic and lumbar spinal cord (T4-L2), and sensory in the dorsal root ganglia of the thoracic spinal cord and the nodose ganglia of the vagus nerve (21). We have examined serial sections of brain, spinal cord, and small intestine from newborn mice inoculated perorally with T3C9 to determine the location of initial virus infection in the nervous system. Neurons of the myenteric plexus of the small intestine immediately adjacent to Peyer's patches were initially infected. Subsequently, viral antigen appeared in the CNS in neurons of the DMNV, indicating that serotype 3 reovirus spreads from the intestinal tract directly to regional nerves and then through parasympathetic fibers to the CNS.

MATERIALS AND METHODS

Virus. Reovirus serotype 3, field isolate strain clone 9 (T3C9; refs. 20 and 22), was derived from laboratory stocks by double plaque purification. Lysates of second-passage stocks grown on L929 mouse fibroblast monolayers were used for virus purification (23).

Titer Determination. Virus titer was determined by standard plaque assay (24). Blood samples were frozen and thawed twice, disrupted by sonication, and assayed in duplicate after serial 1:10 dilutions.

Inoculations. Purified virions were diluted in endotoxin-free saline (for subcutaneous injections) or distilled water (for peroral inoculations) just prior to use. Blue food dye (2 μl/ml) was added to virus suspensions to permit monitoring of the accuracy of inoculations.

Mice were maintained in accordance with the standards outlined in ref. 45 and Public Health Service guidelines. Two-day-old mouse pups born of pregnant NIH Swiss dams (National Cancer Institute, Frederick, MD) were inoculated perorally with purified T3C9 [1×10^8 to 7×10^8 plaque-forming units (pfu)] in 30 μl through a catheter passed into the esophagus (16). Alternatively, pups were inoculated subcutaneously into the forehead with T3C9 (1×10^8 pfu) in 5 μl by using a Hamilton syringe equipped with a 30-gauge needle.

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Abbreviations: CNS, central nervous system; DMNV, dorsal motor nucleus of the vagus nerve; pfu, plaque-forming unit(s).
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Tissue Collection and Preparation. For blood samples, mice were sacrificed by decapitation at specified times after peroral or subcutaneous inoculation with T3C9 (1×10^8 pfu). Blood (50 μl) was collected and diluted in 1 ml of 0.3% gelatin/saline.

On days 2–5 after peroral inoculation, groups of mice were heavily anesthetized by intraperitoneal injection of avertin [100 μl; 1.25% (vol/vol) solution of tribromoethanol/*tert*-amyl alcohol, 2:1 (wt/wt)] and transcardially perfused with buffered saline (pH 7.4), followed by 4% (vol/vol) formaldehyde, and then by 10% (wt/wt) sucrose in buffered saline. Brain and spinal cord with associated dorsal root ganglia were dissected. Spinal cords were frozen immediately in O.C.T. mounting compound (Miles Scientific). Brain tissue was immersed for 24 hr at 4°C in the 10% sucrose solution before freezing at −70°C. On days 2–4 after peroral inoculation, the small intestine was also dissected. The lumen was rinsed with formalin and the tissue was immersed for 30 min in 4% formaldehyde and then for 24 hr at 4°C in 10% sucrose prior to embedding and freezing. Two and 3 days after subcutaneous inoculation, brain tissue was collected as described.

Serial sections of frozen tissue (30 μm, CNS; 16 μm, intestine) were made using a cryostat microtome at −14°C. Sections were collected onto gelatin-coated slides and affixed by air-drying.

Immunohistochemistry. Tissue sections were immersed for 20 min in 1% Triton X-100 in staining buffer (0.15 M NaCl/0.05 M Tris chloride/1 mM Na_2/EDTA/0.05% Tween 20/0.1% bovine serum albumin, pH 7.4). Sections were washed three times and then treated with 0.3% H_2O_2 for 5 min (CNS) or 30 min (intestine) to quench endogenous peroxidase activity. After three washes, sections were stained by a modification of the Vectastain rapid procedure using VectaStain Elite ABC reagents (Vector Laboratories). Each incubation was 25 min at 37°C, with three washes between. The primary antiserum used was a 1:200 dilution of polyclonal rabbit antireovirus affinity-purified antibodies from hyperimmune serum (ref. 25; a combination raised against purified serotype 1 Lang or T3D reovirus). Staining buffer was used for all dilutions and washes. Normal goat serum (1.5%) was added to primary antiserum diluent.

Peroxidase substrate was added to tissue sections for 30–60 sec (a fresh solution of 0.04% diaminobenzidine tetrahydrochloride in 0.1 M Tris chloride (pH 7.6) mixed with an equal volume of 0.05% H_2O_2 in distilled water), and then sections were washed twice in water. CNS sections were counterstained for 2–3 min with 2.5% (vol/vol) Giemsa stain. Cresyl violet acetate or toluidine blue O, each a 0.01% solution in Walpole buffer, pH 4.45 [0.2 M acetic acid/0.2 M sodium acetate, 3:2 (vol/vol)], was used to counterstain intestine sections. Sections were rinsed in water, air-dried, and mounted using Permount (Fisher Scientific).

RESULTS

To obtain evidence for either blood-borne or neural spread of reovirus into the CNS, we cut serial histologic sections through the brain and spinal cord of mice inoculated perorally with T3C9 and stained them by immunoperoxidase for the presence of viral antigen. Viral antigen initially appearing in neurons would indicate direct neural spread from the periphery. Furthermore, the anatomically distinct location of each type of neuronal cell body innervating the small intestine would permit identification of the fiber type supporting transport and replication of virus. Alternatively, virus spreading from the bloodstream into the CNS would infect the meninges surrounding the CNS, endothelial cells lining blood vessels within the CNS, or regions that possess fenestrated endothelium such as the choroid plexus, the hypothalamus, and area postrema. These structures would thus be expected to contain viral antigen if the blood-brain barrier was breached antecedent to neuronal infection.

Viral antigen was not detected in any of the serial sections from the CNS of four mice 3 days after inoculation. In brainstem sections of tissue taken 4 days after inoculation, stained neurons were present bilaterally in the DMNV of two of three animals (Fig. 1*A*). No staining of glial cells, endothelial cells, meninges, choroid plexus, hypothalamus, or area postrema was observed in any animal (data not shown). Viral antigen was not present in thoracic or lumbar spinal cord and dorsal root ganglia, nor in nuclei of the solitary tract, of the mice possessing infected DMNV neurons. This indicates that viral infection of the DMNV preceded demonstrable replication in sympathetic or sensory neurons of the intermediolateral cell column or dorsal root ganglia. Viral antigen was occasionally detected several days later in the meninges, hypothalamus, intermediolateral cell column, and dorsal root ganglia after massive infection of the CNS had occurred, indicating that these cell populations are infectable and/or that viral antigen can be detected in them using our staining procedure.

By 5 days after inoculation, neurons of the DMNV were stained in all four mice examined (Fig. 1*B*), and axons in the vagus nerve tract contained viral antigen (Fig. 1*C*). The

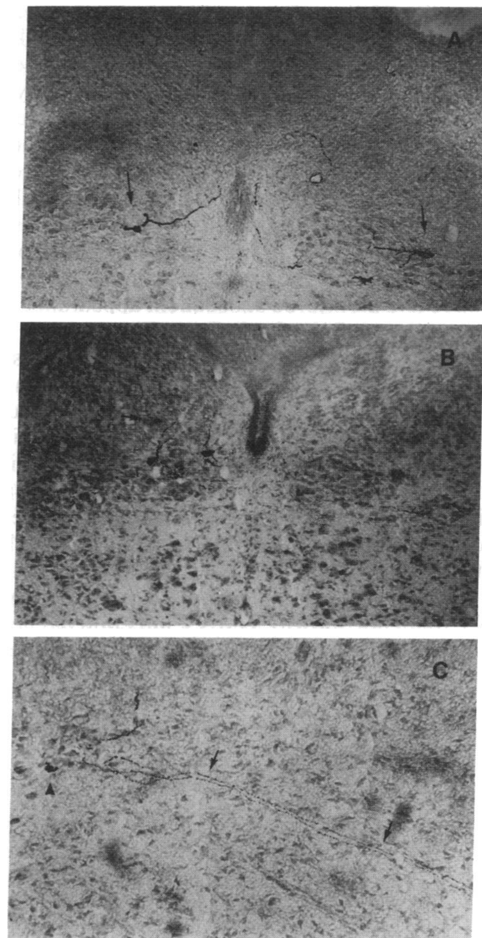

FIG. 1. Transverse brainstem sections of newborn mice inoculated perorally with T3C9. (*A*) Bilateral infected neurons in the DMNV 4 days after inoculation (arrows). (*B*) Bilateral infected neurons in the DMNV 5 days after inoculation and one infected neuron in the left nucleus of the solitary tract (arrow). The hypoglossal nuclei below the DMNV contain no infected neurons. (*C*) Viral antigen in axons within the vagus nerve tract extending from the right DMNV (arrows) with one infected cell body in the nucleus (arrowhead). (×50.)

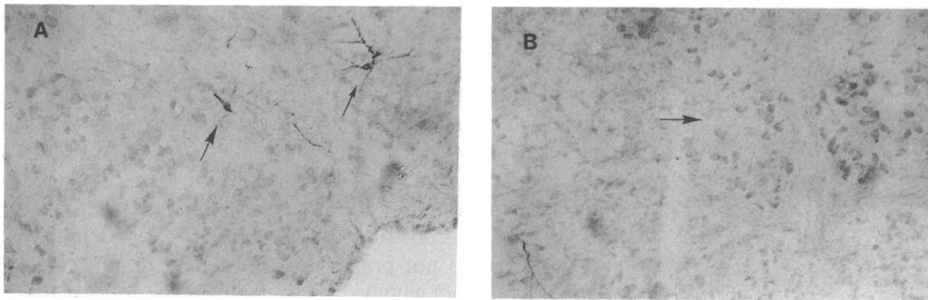

FIG. 2. Transverse brainstem sections of newborn mice inoculated subcutaneously into the forehead with reovirus T3C9. (*A*) Infected neurons in the lateral right facial nerve nucleus (large arrow) and ventral portion of the trigeminal nucleus, pars oralis (small arrow), 3 days after inoculation. (*B*) Absence of staining in neurons of the DMNV from the same mouse (left nucleus indicated by arrow). (×50.)

number of infected neurons was greater than that seen on day 4 but represents a relatively small proportion of the total DMNV neuronal population. Two of four mice had a small number of stained cell bodies in the adjacent nuclei of the solitary tract (Fig. 1*B*). No viral antigen was found in the thoracic, lumbar, or sacral portions of the spinal cords of two mice examined further. Infection of the DMNV indicates that initial penetration of the CNS by reovirus T3C9 occurred by a neural route, specifically along parasympathetic fibers of the vagus nerve. Furthermore, selective infection by a parasympathetic route preceded any detectable infection by sympathetic and sensory routes.

It was possible that the DMNV represented the neuronal population in the CNS most susceptible to T3C9 infection or that parasympathetic fibers of the vagus nerve greatly facilitated viral uptake and transport compared to other neuronal and fiber types. In addition, viremia generated by T3C9 infection of the intestinal tract might, in principle, have resulted in viral spread to other portals of entry into the vagus nerve. Elimination of specific viral transport along the vagus nerve by vagotomy was not feasible to test since the age-related restriction of reovirus replication (13) requires use of newborn mice. Instead, we examined these various possibilities by inoculating T3C9 subcutaneously into the forehead of newborn mice and monitored subsequent appearance of viral antigen in sections of the brainstem. The seventh cranial (facial) nerve and the ophthalmic branch of the fifth sensory (trigeminal) nerve make up the primary innervation of the forehead musculature and skin, respectively. The nuclei of these nerves, in addition to the DMNV, were examined on the same sets of transverse serial sections of the brainstem. Subcutaneous inoculation did, in fact, result in staining of neurons in the brainstem, including those in the nuclei of the facial and trigeminal nerves in all animals (Fig. 2*A*) but not those of the DMNV (Fig. 2*B*). Even a mouse with advanced infection of the brainstem had no antigen-positive neurons in the DMNV (data not shown). DMNV infection did not occur despite a level of viremia as great as that generated after

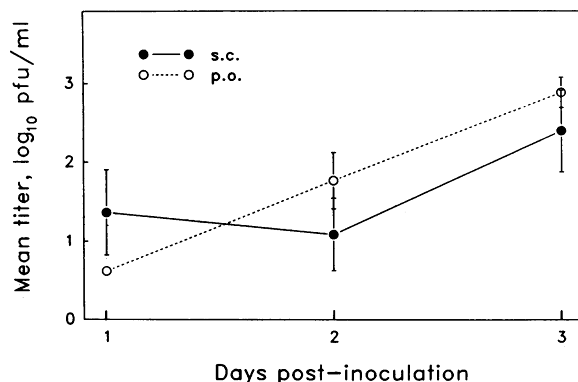

FIG. 3. Titer of virus in the blood of mice inoculated perorally (p.o.) or subcutaneously (s.c.) into the forehead. Blood samples (50 μl) were diluted in 1 ml of gelatin/saline. Plotted points represent the $\log_{10}$ titer from five samples; data are average ± SD.

peroral inoculation, as indicated by the titer of virus present in the blood of cohort animals inoculated either subcutaneously or perorally (Fig. 3). This result established the dependence of DMNV staining on the route of inoculation.

Reovirus inoculated perorally begins to replicate in the intestine and is rapidly disseminated by lymphatic drainage and by the bloodstream within 48 hr of inoculation (Fig. 3 and refs. 15, 16, and 22). Because the vagus nerve sends fibers to many visceral organs in addition to the small intestine, we sought further evidence for direct neural penetration in the small intestine. Transverse serial sections made from the ileum of T3C9-inoculated mice were stained to detect viral antigen. Reovirus-infected mononuclear cells were found 3 days after inoculation distributed at all depths in the lymphoid follicles of the Peyer's patches (Fig. 4*A*). Infected tissue treated with preimmune serum or exposed to peroxidase substrate alone showed no evidence of reaction product (data not shown). Cell bodies between the circular and longitudinal muscle layers in the intestine wall were also stained 3 days (Fig. 4 *A* and *B*) and 4 days (Fig. 4*C*) after inoculation. These were identified as neurons of the myenteric plexus based on cell size, shape, and distribution when compared to neurons visualized by cresyl violet staining (Fig. 4*D*) and by uptake of fluorogold (26). The infected cell bodies apparently were not those of catecholaminergic neurons, as determined by absence of autofluorescence of their fibers in aldehyde-fixed tissue specimens (27). They were found only beneath or adjacent to the margins of ileal Peyer's patches. The small number of infected myenteric plexus neurons observed may explain why only a small proportion of DMNV neurons were infected 1–2 days later.

DISCUSSION

These observations with reovirus strain T3C9 demonstrated that, in the course of a lethal infection§ after gastrointestinal exposure of mice to reovirus, initial spread to the CNS occurred through nerves despite the presence of significant quantities of virus in the bloodstream. Infection of myenteric plexus neurons of the ileum provided evidence for a direct link between entry of T3C9 into Peyer's patches and subsequent infection of DMNV cell bodies in the CNS.

Three observations argue in favor of direct spread to the brainstem from neurons innervating the intestine. (*i*) Myenteric neurons were infected only in plexuses adjacent to ileal Peyer's patches. (*ii*) In the CNS, only the DMNV was initially infected, though virus rapidly disseminates through the bloodstream to muscle and organs not innervated by the vagus. (*iii*) Subcutaneous inoculation of T3C9 in the forehead produced viremia to the same extent as peroral administra-

§LD_{50} of serotype 3 reovirus strains administered perorally is difficult to establish due to the broad dose range over which 30–80% of mice succumb (10^4–10^7 pfu, data not shown). A similar phenomenon occurs in inbred CAF_1 mice and so does not appear to be genetically determined (H. Virgin and K. Tyler, personal communication). The doses used were chosen because they represent an invariably lethal dose and so may promote synchrony of infection.

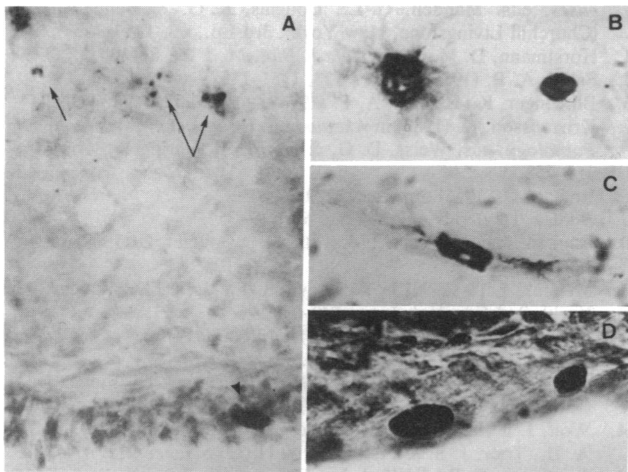

FIG. 4. Transverse sections of distal ileal Peyer's patches from mice inoculated perorally with T3C9. Sections shown were counterstained with toluidine blue O (*A* and *B*) or were not counterstained (*C*). (*A*) Infected mononuclear cells in the lymphoid follicle of an ileal Peyer's patch 3 days after inoculation (arrows) and one infected neuron in the myenteric plexus (arrowhead). (*B*) Two infected myenteric plexus neurons adjacent to a Peyer's patch 3 days after inoculation. (*C*) An infected myenteric plexus neuron 4 days after inoculation. (*D*) Neurons in the myenteric plexus of uninfected tissue resolved with cresyl violet acetate stain. (*A*, ×200; *B–D*, ×315.)

tion but resulted in facial and trigeminal nerve-specific routes of entry into the CNS without evidence of DMNV infection.

We cannot yet distinguish whether infection of neurons in the solitary tract nucleus subsequent to infection in the DMNV represents retrograde transsynaptic spread of virus from the DMNV or spread of virus along vagal sensory fibers from the intestine and then transsynaptically to the nucleus of the solitary tract. We favor the former, however, because no concurrent staining of sensory cell bodies in dorsal root ganglia was observed.

Primary infection of myenteric plexus neurons followed by infection of the DMNV suggests retrograde spread of virus along autonomic (motor) neural paths. Reovirus has been shown to spread retrogradely along somatic motor and sensory fibers from the periphery to the CNS (18, 19) and potentially within the CNS (24) but not to spread along autonomic motor nerves. Other neurotropic viruses can spread through sympathetic (28–31) or parasympathetic (31–33) pathways into the CNS after various routes of peripheral inoculation. Notably, oronasal inoculation of suckling pigs with hemagglutinating encephalomyelitis virus produces infection of the small intestine, vagus nerve, and brainstem; neurons in the submucosal and myenteric plexuses infrequently contain viral antigen (34). However, neither the temporal relationship between infection of these structures nor the brainstem nuclei involved was completely characterized.

The autonomic innervation of the intestinal tract has not been precisely resolved. Two main interconnected neural plexuses lie within the small intestine, the submucosal and the myenteric, each of which receives preganglionic parasympathetic fibers of the vagus nerve and fibers of postganglionic sympathetic nerves. A monosynaptic neural connection between the muscle layer of the small intestine and the brainstem has been demonstrated by appearance of reaction product in DMNV neurons after injection of horseradish peroxidase into rat ileum (35). Pseudorabies virus injected into the stomach wall of rats has also revealed primary infection of DMNV neurons (36). We have extended these observations by demonstrating that an enteric virus, infecting a host by its natural route of entry through Peyer's patches in the small intestine, can gain access to myenteric neurons

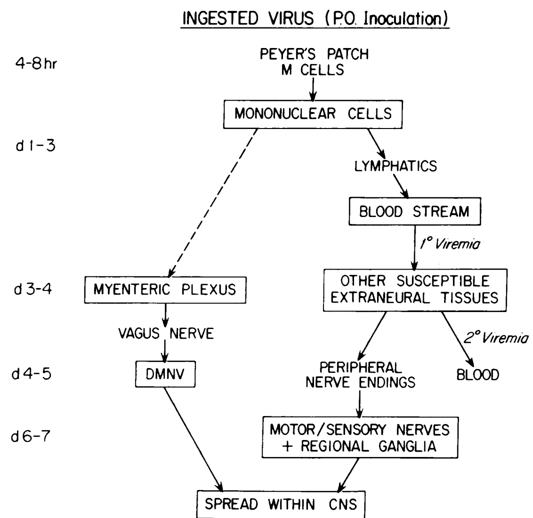

FIG. 5. Schematic representation of steps in reovirus T3C9 pathogenesis. Steps enclosed in boxes indicate information obtained or confirmed in these studies (motor and sensory neurons in spinal cord and ganglia, unpublished observation). Temporal progression is noted at left.

and directly or transsynaptically to preganglionic parasympathetic axon terminals in the intestinal wall that provide a conduit into the CNS.

Our current concept of steps in serotype 3 reovirus pathogenesis that includes our observation of direct neural entry of T3C9 into the CNS is diagrammed in Fig. 5. After virus inoculation, serotype 3 reovirus traverses the intestinal mucosa through M cells overlying ileal Peyer's patches (14). Immunohistochemical observations of T3C9 indicate that a 3-day period of mononuclear cell infection occurs within the patch concomitant with mounting viremia. Three to 4 days after inoculation, viral antigen appears in local neurons of the myenteric plexus. How virus is transmitted preferentially to these neurons, in contrast to submucosal neurons, is not yet known. Transport of T3D within motor and/or sensory nerve fibers occurs by the system of fast axonal transport (18). Transport of T3C9 along the vagus nerve of newborn mice would thus require ≈12 hr (37, 38). With the addition of time required to undergo replication, appearance of antigen in the DMNV would be predicted to occur 1 day after appearance of infection in neurons of the myenteric plexus or 4–5 days after inoculation. The observed delay of overt infection in the CNS would thus reflect the time required for virus replication at each step plus uptake and axonal transport along the vagus nerve.

The observations of initial DMNV infection under circumstances of lethal reovirus infection does not preclude a role for viremia in later stages of CNS infection. By 3–5 days after inoculation, viremia has disseminated T3C9 to muscle and other organs (L.A.M., unpublished data). Ensuing replication may permit virus entry into peripheral nerves at these multiple points (5–7), thus eventually contributing a second wave of CNS invasion through nerves (Fig. 5). Spinal cord sections from mice 6–7 days after inoculation with T3C9 contained profuse infected motor neurons (L.A.M. and R.L.S., unpublished data). Secondary infection of extraneural tissues may eventually promote sufficient viremia to permit CNS invasion by hematogenous routes as well.

An IgG monoclonal antibody directed against the reovirus serotype 3 cell attachment protein has been shown to reduce viremia (20) and protect mice from lethal infection with T3D (25). However, T3D is known to spread through nerves from the footpad (18, 19), not by the bloodstream. Significantly, this antibody blocks penetration of the nervous system by

T3D and transmission of virus within the CNS after neuronal infection has commenced. Antibody also prevents T3C9 infection of the CNS after peroral or footpad inoculation (20). Thus termination of viremia and prevention of CNS infection by antibody cannot be considered direct evidence of blood-borne invasion of the nervous system in view of the demonstrated capacity of antibody to abort virus spread to and within the nervous system.

The capacity of passively administered or vaccine-induced serum antibody to eliminate viremia and block CNS infection has figured prominently in the general acceptance of hematogenous spread of poliovirus to the CNS (1–4, 8, 9, 11, 12). However, little direct evidence exists for blood-borne penetration of the CNS by poliovirus (9, 10). Many features of poliovirus pathogenesis (for review, see refs. 1–5, 8) are similar to our observations with reovirus T3C9. Poliovirus undergoes primary replication in the alimentary tract, including ileal Peyer's patches. Virus can be isolated from the bloodstream of humans and experimental animals soon after alimentary exposure, and viremia generally precedes CNS invasion. However, poliovirus is known to spread through neuronal pathways once within the CNS and in peripheral nerves after experimental inoculation. Severe infection of brainstem nuclei, most often the vagus nucleus, has been observed in fatal cases of bulbar poliomyelitis in humans (39) and in chimpanzees and monkeys fed poliovirus. Lastly, the temporal events of reovirus infection correspond well to analyses of natural poliovirus infection (40, 41), allowing for increased transport time within the longer axons of humans. Although both poliovirus and reovirus mount a viremia that can be terminated by antiviral antibody, evidence from reovirus that antibody can prevent neuronal infection (20) and that T3C9 spreads to the CNS through nerves from the intestinal tract supports the concept of neural spread of poliovirus to the CNS after extraneural replication (5–7). Whether poliovirus and encephalitic strains of other enteric viruses do, in fact, penetrate the CNS by means similar to reovirus awaits further investigation. In this regard, uptake of virus into lymphoid tissue of the alimentary tract [as shown for reovirus (14), subsequently for poliovirus (42), and potentially other viruses (34, 43, 44)] may represent a ubiquitous means of enteric virus entry into the host. Access to neurons in the alimentary tract plexuses may likewise be a common feature of these infections and thus a common route of CNS penetration.

Our observations with reovirus T3C9 identify a series of distinct events in the pathogenesis of encephalitic infection, tracing its path from the intestinal lumen to peripheral nerve entry in the myenteric plexus and initial entry into the CNS. This knowledge may facilitate study of the individual steps in this process, such as the site at which serum antibody blocks neuronal infection. It also raises the question of how virus is transmitted from lymphoid follicles of the Peyer's patch to neurons of the myenteric plexus, and ultimately the consideration of interaction between neurons and elements of the immune system.

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