Oral Inoculation with Herpes Simplex Virus Type 1 Infects Enteric Neurons and Mucosal Nerve Fibers within the Gastrointestinal Tract in Mice

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Herpes simplex virus type 1 (HSV-1) is commonly encountered first during childhood as an oral infection. After this initial infection resolves, the virus remains in a latent form within innervating sensory ganglia for the life of the host. We have previously shown, using a murine model, that HSV-1 placed within the lumen of the esophagus gains access to nerves within the gut wall and establishes a latent infection in sensory ganglia (nodose ganglia) of the tenth cranial nerve (R. M. Gesser, T. Valyi-Nagy, S. M. Altschuler, and N. W. Fraser, J. Gen. Virol. 75:2379–2386, 1994). Peripheral processes of neurons in these ganglia travel through the vagus nerve and function as primary sensory receptors in most of the gastrointestinal tract, relaying information from the gut wall and mucosal surface to secondary neurons within the brain stem. In the work described here, we further examined the spread of HSV-1 through the enteric nervous system after oral inoculation. By immunohistochemistry, HSV-1 was found to infect myenteric ganglia in Auerbach's plexus between the inner and outer muscle layers of the gut wall, submucosal ganglia (Meisner's plexus), and periglandular ganglion plexuses surrounding submucosal glands. Virus-infected nerve fibers were also seen projecting through the mucosal layer to interact directly with surface epithelial cells. These intramucosal nerve fibers may be a conduit by which intraluminal virus is able to gain access to the enteric nervous system from the gastrointestinal lumen.

Oral infection with herpes simplex virus type 1 (HSV-1) during childhood causes either an asymptomatic infection or an acute infection of the gums, oral mucosa, and tongue termed gingivostomatitis (7, 12, 37). That a considerable percentage of the population is exposed to HSV-1 early in life is attested to by seroepidemiologic studies which demonstrate rising levels of seropositivity for HSV during childhood, with 70 to 90% positivity by adulthood (7, 11, 25, 37). Like other alphaherpesviruses (pseudorabies virus, varicella-zoster virus, and bovine herpesvirus), HSV-1 exhibits tropism for the peripheral processes of nerves which innervate the body surface (34, 35). Upon entering a nerve ending, the virus is actively transported to the nerve cell nucleus located in sensory ganglia, often a considerable distance away from the initial site of inoculation (10). Following an acute infection in these ganglia, the HSV-1 genome may remain in a latent form within the nuclei of sensory neurons for the life of the host or periodically reactivate to cause recrudescent disease or asymptomatic shedding at the body's surface. While HSV-1 is most commonly thought to latently infect the trigeminal ganglion of the fifth cranial nerve innervating the face and oral mucosa (2, 3), latent HSV-1 has also been found in humans in the nodose ganglia innervating the gastrointestinal tract (36). Furthermore, the virus is a frequent cause of recurrent esophageal mucosal disease in humans, particularly those who are immunocompromised (1, 8, 13, 26, 38). We have recently shown that HSV-1, orally inoculated into the esophageal lumen of mice, travels to

the sensory ganglion (nodose ganglion) of the vagus or tenth cranial nerve, where it establishes a latent infection (17). This discovery led us to believe that HSV-1 may also be an enterically acquired pathogen that is able to breach the mucosal surface of the gut to encounter nerve fibers of the enteric nervous system.

The nervous system of the gastrointestinal tract consists of a complex intrinsic network of interconnected neurons within the gut wall itself (enteric nervous system) (reviewed in reference 15). These vast numbers of enteric neurons direct movement, secretions, and blood flow within the alimentary tract and are regulated by local reflex pathways within the gut as well as by extrinsic innervation from sympathetic, spinal, and vagal parasympathetic nerves. Primary sensory neurons in the nodose ganglia, responding to different sensory modalities, send afferent fibers via the vagus nerve to most of the gastrointestinal wall. Mechanoreceptors (sensitive to stroking), chemoreceptors (responsive to acidity, hypertonicity, and intraluminal chemical composition), and thermoreceptors have been localized to the gut mucosa by functional studies; mechanoreceptors are also found in the muscular and serosal layers throughout the gastrointestinal tract (reviewed in references 19, 28, and 29). Via these receptors, vagal sensory pathways are able to respond to and modify changes in the gastrointestinal lumen.

In mice after oral inoculation, HSV-1 infects myenteric and nodose ganglia and spreads to secondary sensory neurons (to which the nodose ganglia centrally project) within the nucleus tractus solitarius of the medulla (17, 18). Because HSV-1 delivered intraluminally to mice appears to target neurons of the enteric nervous system and preferentially spread to, infect, and establish latency in neurons of the vagus nerve sensory pathways, we questioned whether terminal vagus nerve fibers were involved in the spread of HSV-1 after oral inoculation. In the

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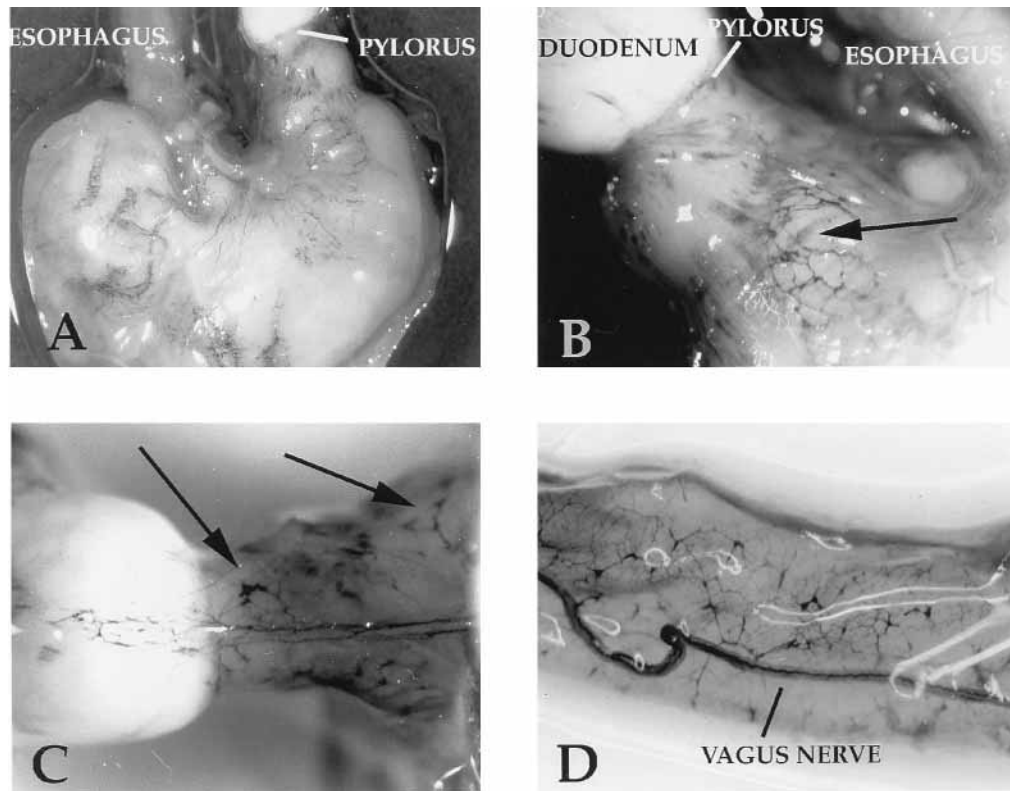
FIG. 1. Whole-organ indirect immunohistochemistry. The esophagus, stomach, and proximal duodenum were reacted with rabbit polyclonal HSV-1 antiserum (Dako) and then with peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride as described in reference 18. HSV-1-infected areas are identified by dark staining. (A to C) Tissue from a BALB/c mouse 6 days after oral inoculation with HSV-1 strain 17+. (A) Dorsal view of the stomach, with the attached esophagus and duodenum, showing virus infection in penetrating gastric nerves and the body of the stomach and a concentration of infection in the prepyloric region. (B and C) Enlarged views of the gastroduodenal junction. The arrows point to infected myenteric ganglia and internodal fibers within the gastric antrum. In panel C (a view along the greater curvature of the stomach), infected penetrating nerves are apparent in the mesenteric attachment of the stomach and proximal duodenum. (D) At 8 days p.i. with HSV-1 strain 17+, the esophagus of a SCID mouse has widespread viral infection in myenteric ganglia and interconnecting fibers. Also shown are infected fibers of the vagus nerve.

present study, using immunohistochemistry, we further characterize the spread of HSV-1 within neurons of the gut wall. We show that after the placement of HSV-1 into the esophageal lumen of mice, the virus spreads via internodal strands of the enteric nervous system, infecting neurons of the myenteric, submucosal, and periglandular plexuses of the esophagus, stomach, and duodenum, without significant spread to surrounding tissues. HSV-1-labeled terminal nerve fibers were also seen penetrating into the lamina propria of the gastric and duodenal mucosa to interact directly with surface epithelial cells. These new findings in immunocompetent hosts indicate that HSV-1, previously not considered an enteric pathogen, spreads considerably within all levels of the enteric nervous system, including nerve fibers directly in contact with the mucosal epithelium. This discovery raises questions regarding a possible role for HSVs in inflammatory mucosal and functional disorders of the human gastrointestinal tract.

HSV-1 infects enteric ganglia after oral inoculation. In a previously published study of immunocompetent BALB/c mice orally inoculated with neurovirulent HSV-1 strain 17+, infected myenteric ganglia were seen in the esophagus and stomach 4 days postinoculation (p.i.). By immunohistochemistry with thin tissue sections (5 to 6 μm), these infected ganglia were found to represent a small percentage of the total ganglia examined; no infected epithelial cells were apparent (17). Further work with a severely attenuated HSV-1 strain (*in*1814) in immunocompromised severe combined immunodeficiency

(SCID) mice again showed a limited propensity for the virus to infect mucosal epithelial cells, despite widespread persistent infection of neurons and support cells in the enteric nervous system. These hosts were unable to clear the primary acute infection, and yet even in the absence of specific host immunity, viral spread in these animals was limited primarily to cells of the enteric and vagal parasympathetic nervous system (18).

In the present study, in order to better visualize viral spread in the gut, we used a high-titer inoculum of wild-type strains F or 17+ and applied the technique of whole-organ immunohistochemistry to further analyze the entire esophagus, stomach, and proximal duodenum after intraesophageal inoculation. As described elsewhere (18), this technique allows gross detection of viral protein expression sites in intact tissues with the aid of a dissecting microscope. Immunostained sites can then be selected for further histological examination. Controls, consisting of uninfected tissues, and acutely HSV-1-infected tissues processed without primary HSV-1 antiserum were consistently negative.

BALB/c mice received either 1×10^6 ($n = 13$), 3×10^6 ($n = 15$), or 5×10^6 ($n = 3$) PFU of wild-type HSV-1 strain 17+ or F in 200 μl of serum-free medium delivered by intraesophageal cannulation as previously described (18). HSV-1 immunostaining was first apparent in fibers of the cervical, thoracic, and abdominal portion of the vagus nerve beginning 4 days p.i. With polyclonal HSV-1 antiserum (Dako, Carpinteria, Calif.) being used, virus-infected nerve fibers were visible along the

mesenteric border and in nerves penetrating the serosal surface of the gut. Infected enteric ganglia were also first noted at 4 days p.i. Initially, HSV-1 antigen-stained ganglia were most prominent along the lesser curvature of the gastric antrum, extending caudally to include the gastroduodenal junction (Fig. 1A to C). Over the following days, up until 9 days p.i., HSV-1 infection in the enteric nervous system continued to progress, involving additional ganglia and internodal strands throughout the stomach and proximal duodenum. In immunocompetent BALB/c mice, symptoms of this acute infection resolved by 9 days p.i. or the animals died of encephalitis within 2 weeks of the inoculation. Animals that died 12 to 14 days after the inoculation had no evidence of HSV-1 in the gastrointestinal tract (presumably cleared by the immune system), whereas those that died within the first week p.i. had widespread HSV-1 infection in the enteric nervous system of the esophagus, stomach, and proximal small intestine, as detected by immunohistochemistry. When SCID mice ($n = 22$) were orally infected with HSV-1 strain 17 or F, enteric nervous system infection was once again first apparent 4 days p.i. This infection subsequently spread to involve enteric ganglia in most of the stomach, proximal duodenum, and esophagus (Fig. 1D). All SCID animals that succumbed to this infection had widely disseminated enteric HSV-1 infection at the time of death (usually within 10 days p.i.). Some mice, both normal and immunocompromised, remained well after oral inoculation, however, without any evidence of enteric infection. We take this to indicate that intraluminal inoculation is a relatively inefficient route of infection for HSV-1, with a considerable portion of the inoculum being inactivated by such nonspecific host defenses as luminal pH, digestive enzymes, and mucosal barriers to infection (18).

The myenteric (Auerbach's) plexus is an interconnected network of nerve strands and small ganglia (each containing an average of 40 neurons) located between the external longitudinal and circular muscle coats of the gastrointestinal tract. Meissner's submucous plexus is found just below the gut mucosa. Compared with those of the myenteric plexus, the ganglia of the submucous plexus are smaller and contain fewer nerve cells; their interconnecting (internodal) strands also have fewer nerve fibers. Both plexuses are continuous around the circumference and along the length of the alimentary tract, essentially forming sheets of intercommunicating neuronal networks running parallel to the gut lumen (reviewed in reference 15). After intraesophageal inoculation, the myenteric and submucosal plexuses of the stomach and duodenum were infected with HSV-1. In whole-mount preparations, infected neurons and supporting glial cells were clearly apparent. Infected nerve fibers were seen within myenteric ganglia, traveling through internodal strands to communicate with additional ganglia, and in groups of nerve bundles within the muscle coats (Fig. 2). Both uninfected and heavily infected neurons were seen simultaneously within the same enteric ganglion, indicating to us that spread within the ganglia proceeds most likely by specific transneuronal spread rather than by adjacent cell-to-cell spread. This mode of spread was also made apparent by the lack of infectious spread from cells of the nervous system to other tissues of the gastrointestinal wall (17, 18) and was further underscored by the work of others demonstrating the effectiveness of herpesviruses as specific transsynaptic tracing agents (6, 9, 20, 21, 30, 31, 33).

Thick tissue sections also revealed diffuse HSV-1 infection of the submucosal plexus in the gastric wall, particularly in neurons and nerve fibers enveloping the gastric glands and in duodenal glands concentrated around the pylorus (Fig. 2A and 3). Within the duodenum, infected neurons were seen imme-
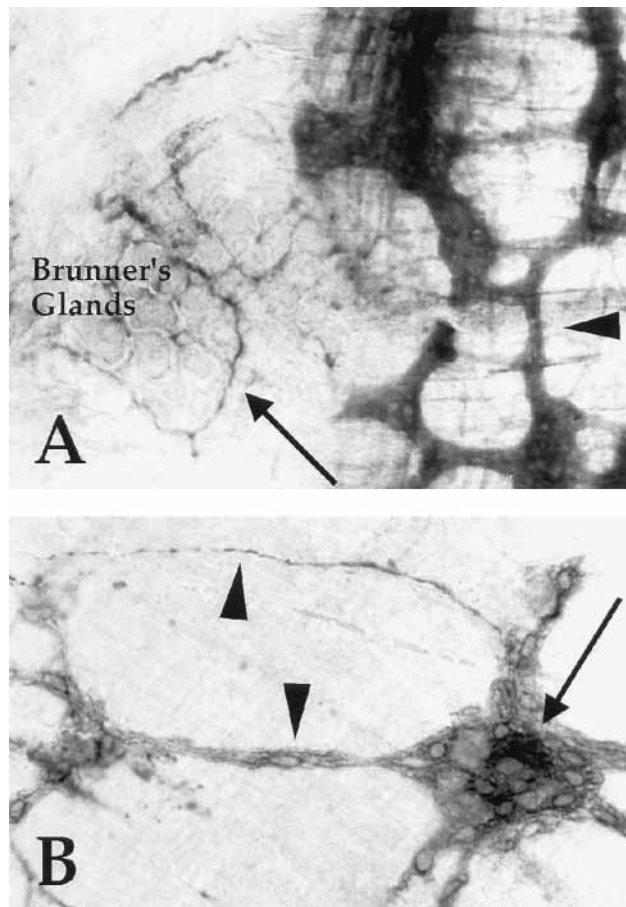

FIG. 2. HSV-1-infected enteric nerves and ganglia in the duodenum of orally infected mice. The tissues are sectioned parallel to the mucosal surface. (A) A SCID mouse at 8 days p.i. with strain 17+. Two layers within the intestinal wall are shown. To the right are infected myenteric ganglia and internodal fibers (arrowhead) within Auerbach's plexus between the outer muscular walls; to the left are virus-infected nerve fibers (arrow) extending from the deeper myenteric plexus to surround duodenal Brunner's glands in the submucosa. Magnification, ×100. (B) Whole mount of myenteric ganglia of a BALB/c mouse at 6 days p.i. with HSV-1 strain F. Within an enteric ganglion are both infected (black arrow) and uninfected (white arrow) neurons. Infected internodal nerve fibers (arrowheads) are shown extending towards other ganglia. Magnification, ×400.

diately adjacent to Brunner's glands in the submucosa: the processes of these nerves wove in between and just below the glandular epithelium, often surrounding a cluster of glands. A cross section of the duodenum demonstrated infected submucosal ganglia with connecting internodal strands spreading circumferentially around the intestinal wall, linking infected neurons and ganglia (Fig. 3A to D).

HSV-1 labels intramucosal nerve fibers and associated cells. HSV-1-labeled terminal nerve fibers penetrated into the lamina propria of the gastric and duodenal mucosa and into the mucosa of the esophagus. In the stomach and duodenum, the bulk of this activity was centered about the gastroduodenal junction as demarcated by the pyloric sphincter (Fig. 3A and B). In the distal antrum and pyloric region of the stomach, immunostained fibers extended to reach the basal surface of luminal epithelial cells. In thick tissue sections or whole mounts, these mucosal fibers appeared to connect to submucosal ganglia directly below and with adjacent infected submucosal ganglia via internodal fibers. As was seen in the gastric mucosa, infected nerve fibers in the duodenum also extended
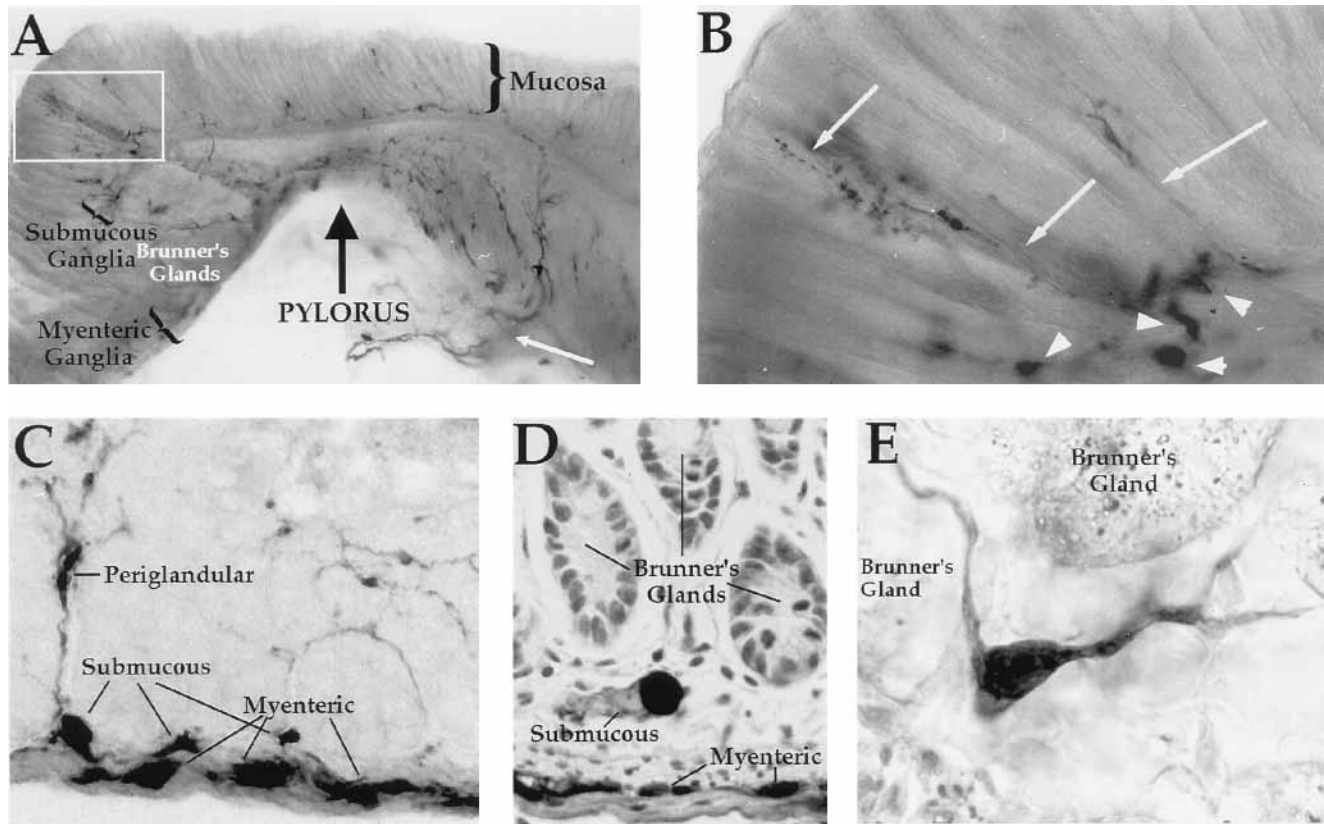
FIG. 3. HSV-1 immunostaining of the gastroduodenal junction. (A and B) Whole mounts of the pyloric region from a BALB/c mouse at 7 days p.i. with strain 17+. In panel A, the stomach is to the right of the pylorus and the duodenum is to the left. HSV-1-infected myenteric and submucosal ganglia and fibers (staining black) are shown. Also shown are infected penetrating nerves (white arrow) carried along the mesentery and infected nerves penetrating the pyloric sphincter of the stomach. In panel B, which is the area enclosed in the box in panel A enlarged, infected submucosal ganglia (arrowheads) and infected nerve fibers penetrating into the duodenal mucosa (arrows) are shown. Magnification, ×40 (A) and ×200 (B). (C) A section (25 μm thick) of proximal duodenum from a BALB/c mouse at 5 days p.i. with strain F showing HSV-1-infected ganglia and nerve fibers in the area of duodenal Brunner's glands. Magnification, ×200. (D) A section (7 μm thick) showing HSV-1-infected myenteric and submucosal ganglia in the duodenum. Magnification, ×200. (E) A section (25 μm thick) showing an infected neuron adjacent to Brunner's glands in the submucosa. Magnification, ×1,000.

towards the luminal epithelial layer. In the proximal duodenum, single and occasionally multiple infected nerve fibers were seen spreading towards the tip of an individual duodenal villus (Fig. 4). Often these fibers terminated on a single infected mucosal epithelial cell; less often, clusters of infected epithelial cells were seen around terminal nerve fibers. Within the villus lamina propria, labeled nerve fibers were found in contact with virus-infected spindle-shaped cells. These spindle cells, characterized by an oval nucleus and several slender dendritic-like processes, were identical to cells that Berthoud et al. (4) described as being associated with vagus nerve sensory receptors originating from neurons in the nodose ganglia. Thus, it is likely that the infected nerve terminals we are describing are sensory and also have their origins in the nodose ganglia.

Vagal sensory receptors are known to respond to intraluminal changes, transmitting information throughout the enteric and parasympathetic nervous system; they may also be involved in the uptake and dissemination of orally acquired neurotropic herpesviruses. We describe here how after intraesophageal inoculation, HSV-1 spreads to infect multiple layers of the intrinsic enteric nervous system and to infect mucosal nerve fibers believed to be vagal sensory receptors. Peripheral sensory receptors in the gut consist of free nerve endings rather than specialized receptors, as found elsewhere in the body (5, 16, 22, 23, 27). It has long been questioned whether these

mucosal afferent fibers interact directly with luminal epithelial cells and whether sensory nerve endings can directly sample the intraluminal contents. Recently, Berthoud et al. have shown vagus nerve sensory endings in the rat duodenal mucosa by injecting the neurotracer DiI directly into the nodose ganglia (4). These vagal afferent terminal fibers, originating from sensory neurons in the nodose ganglia, were found to connect with enteric ganglia in both the myenteric and submucous plexuses and to arborize terminally within the villus lamina propria in close contact with surface epithelial cells. It has been suggested that these terminal nerve endings, positioned as they are, may be able to directly sample the lumen or relay information from surface epithelial cells and essentially function like taste cells of the luminal contents. Similarly, peripheral vagal afferent receptors have also been identified in the gastric mucosa, concentrated around the prepyloric region (22, 27).

We previously showed in immunodeficient (SCID) animals that HSV-1 spread in the nervous system is determined by the innervation of the initial inoculation site and that the virus does not spread indiscriminately, for example, by the circulation (18). It is highly likely that virus inoculated in the esophageal lumen also reaches the mucosa of the stomach and small intestine, where it can apparently access the enteric nerve network; in immunocompetent mice, we have seen significant viral infection in the pyloric region without any apparent infection of the esophageal enteric nerves. The finding of Bud-
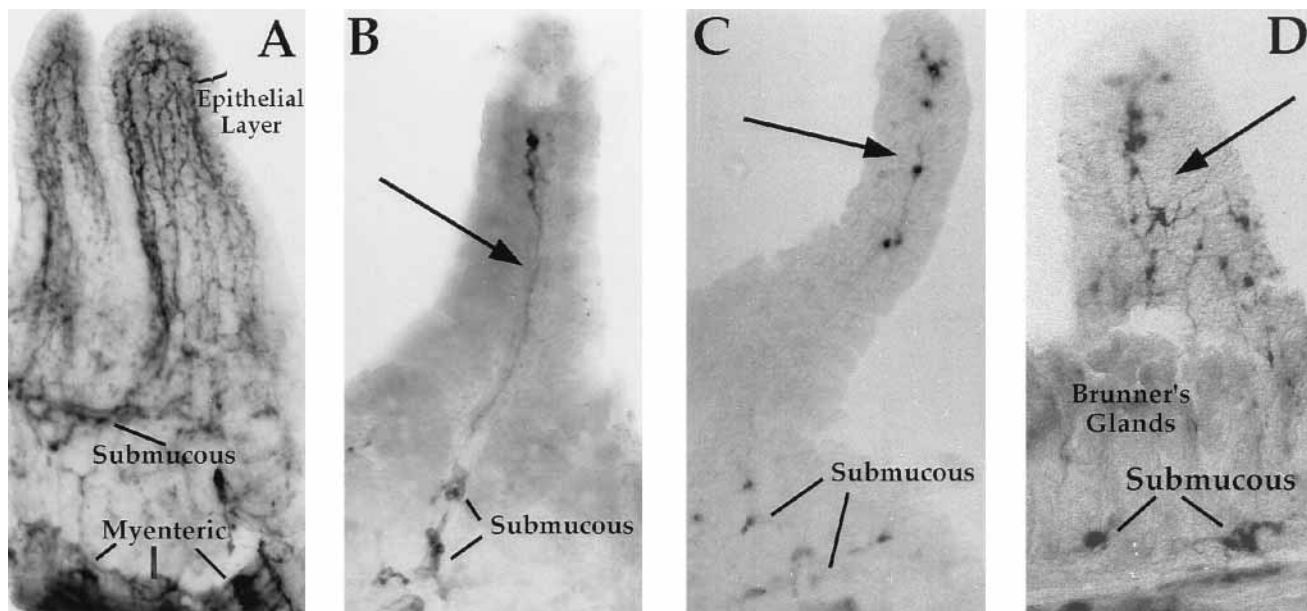
FIG. 4. Individual duodenal villi dissected out and immunostained for either PGP 9.5 or HSV-1 antigens. (A) Uninfected duodenum reacted with a neuron-specific antibody against PGP 9.5 (Dako) (14, 32), which stains myenteric and submucosal ganglia and interconnecting neuronal fibers, including an elaborate web of nerve fibers in the lamina propria just below the epithelial surface. (B to D) Duodenal villi from virus-infected mice immunostained with polyclonal HSV-1 antiserum. Infected submucosal ganglia and neuronal fibers extending into the lamina propria of intestinal villi (arrows) are shown. Magnification (for all panels), ×400.

dingh et al. that infectious virus is shed rectally for weeks after symptomatic gingivostomatitis in children (7) lends further support to this observation. Virus may breach the mucosal surface in a number of places in the gut. Once inside, however, it seems apparent that at least regional transsynaptic viral spread occurs throughout all layers of the intrinsic enteric nervous system. Using a neurotropic reovirus strain, Morrison et al. described the spread, after oral inoculation, to vagal efferent areas of the brain stem (dorsal motor nucleus) (24). HSV-1 also spreads centripetally to vagal areas of the brain stem, including the dorsal motor nucleus after oral inoculation (17, 18). However, during early HSV-1 infection, we clearly see a preference for sensory fibers which originate in the nodose ganglia and terminate centrally in the nucleus tractus solitarius of the brain stem. Furthermore, in the gut, Morrison et al. found reovirus-infected neurons only within myenteric ganglia immediately adjacent to infected lymphoid follicles (Peyer's patches) in the ileum, whereas we find HSV-1-infected enteric neurons predominantly in the proximal duodenum (where Peyer's patches are absent) and throughout all levels of the gut wall, including the surface mucosa. This finding suggests to us that in the gastrointestinal tract, neurotropic viruses may utilize both efferent and afferent fibers which terminate in the viscera.

We were surprised to discover that relative to enteric neurons, the gastrointestinal epithelium was much less likely to be acutely infected with HSV-1 (17, 18). This result may reflect an inherently lower susceptibility of these cells to acute infection or a limited ability of HSV-1 to spread cell to cell within the surface epithelium. Either of these explanations, coupled with the rapid turnover of infected gut epithelial cells, probably accounts for the paucity of HSV-1 immunoreactive surface cells in both normal and immunocompromised mice. Recently, we described the development of erosive gastric and esophageal mucosal ulcers in SCID mice following intraesophageal inoculation with an attenuated HSV-1 strain (18). Despite a persistent and widespread viral enteric nervous system infec-

tion, the ulcers in these animals were not directly infected; rather, they were found to overlay virus-infected enteric ganglia. This result suggests that chronic enteric nerve dysfunction or inflammation may alter the natural mucosal barrier of the gut, ultimately resulting in epithelial disintegration and ulceration. In less dramatic ways, acute, latent, or reactivated HSV-1 enteric nervous system infection may also be involved in the pathogenesis of chronic, recurrent functional human gastrointestinal disorders. The infected neuronal networks which we describe here are likely to contribute to such physiologic disorders.

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