Latent Herpes Simplex Virus Type 1 Gene Expression in Ganglia Innervating the Human Gastrointestinal Tract

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Using in situ hybridization, we demonstrated that latent herpes simplex virus type 1 (HSV-1) gene expression is prevalent in human adult nodose ganglia. This suggests that infection of gastrointestinal sensory nerves, probably through swallowed virus-laden oral secretions, occurs commonly and that HSV-1 reactivating from this site may play a role in recurrent gastrointestinal disorders.

Striking characteristics of the ubiquitous alpha group of herpesviruses are a tropism for the peripheral processes of sensory nerves and the ability to remain latent in neurons despite a specific host immune response (28). Latent herpes simplex virus type 1 (HSV-1) has principally been isolated from human trigeminal sensory ganglia (3, 4, 35). Recurrent disease of the mouth (herpes labialis) or eye (herpes keratitis) occurs when the virus reactivates and travels down branches of the trigeminal nerve to cause a peripheral infection with characteristic lesions (20, 31, 37).

HSV-1 also causes esophageal disease and has been associated with gastrooduodenal lesions (1, 6, 10, 22). Furthermore, Warren et al., while reporting on the prevalence of HSV-1 latency in human neurons innervating the head and neck, isolated HSV-1 from a human nodose ganglion (34). The nodose ganglia, located just outside the jugular foramen, contain afferent neurons which form part of the vagus, or Xth cranial, nerve. Axons from these sensory neurons travel via the glossopharyngeal (IX) and vagus (X) nerves to innervate the thoracic viscera and alimentary canal from the pharynx to the colon (2). It is known that during both primary and reactivated HSV-1 infection, infectious virus is swallowed and is excreted in feces (5). Recently, we have shown that HSV-1 placed into the lumen of the proximal esophagus of mice gains access to nerves in the gastrointestinal wall and is transported to the nodose ganglia, where ultimately a long-term latent infection is established (16). We suggest that a similar pattern of spread may occur in humans during oral HSV-1 infection. From seroepidemiologic studies, it is known that up to 90% of the population is seropositive for HSV-1 by adulthood (7, 25). If recurrent gastrointestinal disorders are caused, as are herpes labialis and keratitis, by HSV-1 reactivation, latent virus should be commonly (based on the high seroprevalence rate) found in sensory neurons innervating the bowel surface.

During HSV-1 latency in neurons, gene expression (as determined by in situ hybridization) is restricted to one strand of an 8.3-kb region within the long repeat elements of the 150-kb double-stranded viral DNA genome (Fig. 1A) (9, 24, 29, 38). Transcription from this region results in a number of viral RNAs which have been called the latency-associated transcripts (LATs) (33). The major LAT expressed during latency is an abundant (up to 1,000 molecules per infected cell) 2.0-kb

RNA which localizes to the nuclei of infected neurons. Although the specific function of these RNA molecules has yet to be determined, their presence in neuronal nuclei is an established marker for latent HSV infection (8, 14, 32, 36). In the work described here, we used in situ hybridization for the LATs to test for HSV-1 latency in human nodose ganglia.

In situ hybridization. Twenty-one human nodose ganglia were obtained from 11 adult cadavers that had been embalmed in 6.8% formalin within 24 to 48 h of death. None of the subjects died from herpesvirus-related causes. The nodose ganglion was identified as a swelling in the vagus nerve as it exits the jugular foramen. Under a dissection microscope, the superior laryngeal nerve and the hypoglossal (XII) nerve were used to define the caudal and rostral extent of the ganglion. Ganglia were embedded in paraffin such that each 5- to 7- μ m-thick section contained 12 to 16 pieces from regions throughout the ganglion. Twenty-four to 48 total sections from both the right and left ganglia of all subjects, except subject 21 (only one ganglion was available), were examined. Because HSV serology was unknown in these subjects, nodose ganglia obtained at autopsy from HSV-seronegative children served as negative controls.

Linearized plasmids and digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, Ind.) were used to transcribe RNA strand-specific LAT probes (Fig. 1). Slide-mounted tissues were hybridized (60 to 120 pg of probe/ μ l) overnight at 60°C as described previously (17), except that detection was accomplished with alkaline phosphatase-conjugated anti-digoxigenin antibody as specified by the Genius Detection System (Boehringer Mannheim) with modification. RNase-pre-treated slides were used as controls. Murine sensory ganglia (trigeminal and nodose) uninfected, latently infected, and acutely infected with HSV-1 were used to prove the sensitivity and specificity of the riboprobes. Both the 901- and 437-nucleotide (nt) antisense probes (LAT 1 and 2, respectively) hybridized specifically to murine ganglia latently infected with HSV-1 (Fig. 2A and B); the opposite strand probes did not. Neither the sense nor the antisense HSV-1 probes hybridized with uninfected murine ganglia or with ganglia from HSV-seronegative human controls.

HSV-1 LAT antisense probes. The 901-nt LAT 1 probe hybridized to ganglia from 9 of 11 (82%) individuals, usually bilaterally. Sixteen of 21 ganglia (76%) tested positive (Table 1). This probe, which is homologous to the major 2.0-kb LAT and to sequences immediately upstream of the 2.0-kb LAT (termed the minor LAT [14]), produced a strong hybridization signal in the nuclei of ganglionic neurons. The same tissues

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FIG. 1. Origin of riboprobes used for in situ hybridization. (A) Schematic of the 150-kb HSV-1 DNA genome. The major LAT is a 2.0-kb transcript from within a larger approximately 8.3-kb LAT region. The immediate-early gene *ICP0* and the late gene *ICP34.5* are transcribed from the strand opposite the LAT. LAT-containing genomic fragments were subcloned into the *EcoRV* site of plasmid Bluescript KS (Stratagene, La Jolla, Calif.), and the orientation was determined by restriction endonuclease digestion. TR_L, terminal long repeat; UL, unique long region; IR_L, inverted long repeat; IR_S, inverted short repeat; US, unique short region; TR_S, terminal short repeat. (B) LAT 1 probe. Transcription from the T3 promoter of pBS-BST1 generated a 901-nt LAT antisense probe. (C) LAT 2 probe. Transcription from the T3 promoter of pBS-HS generated a 437-nt antisense major LAT probe which did not overlap the LAT 1 probe. T7 transcription generated a probe which hybridized to 215 bp of the 3' end of *ICP0*. The numbers in parentheses refer to the McGeoch sequence (23). B, *BstE*; H, *HpaI*; S, *SalI*; H, *HhaI*; X, *XhoI*; K, *KpnI*; Hd, *HindIII*; X, *XhoI*; Bs, *BsaI*.

were also examined with the 437-nt LAT 2 probe, generated from sequences entirely within the 2.0-kb LAT region. Human nodose ganglia which were positive with the LAT 1 probe hybridized strongly to the second probe. On adjacent slide sections (one reacted with the LAT 1 probe, the other reacted with the LAT 2 probe), nuclei from the same neuron were found to hybridize specifically to both of these antisense probes; neurons negative with one of the LAT probes also failed to hybridize to the other probe (Fig. 2D to G). Hybridization was found in the nuclei of both small- and large-diameter neurons throughout the ganglia, including the caudal pole. The frequency of positive neurons in a single ganglion ranged from 0.02 to 2.1%. This percentage of infected neurons is similar to the range reported for HSV-1 latently infected human trigeminal ganglia (0.2 to 4.3%) (8) and demonstrates for the first time that acquisition of latent viral genomes in these two ganglia occurs to a similar degree. Neither the LAT 1 probe nor the LAT 2 probe hybridized to nodose ganglia obtained from HSV-seronegative human controls (Fig. 2C).

HSV-1 LAT sense probes. When human nodose ganglia were hybridized with probes generated from the strand opposite the LAT 1 and 2 probes (sense probes), no signal was detected. Figure 3 shows nodose ganglion neurons specifically hybridizing to both the LAT 1 and LAT 2 antisense probes but not hybridizing to either of the sense strand probes. The sense LAT 2 probe, which hybridizes to acutely infected murine ganglia (not shown), overlaps the 3' end of HSV-1 immediate-early gene *ICP0* by 215 nt. This gene is expressed within minutes of initiation of the virus replicative cycle, and it is believed to be involved in the early stage of reactivation from latency (13, 19, 21). The absence of *ICP0* expression in these tissues is evidence against the presence of replicating virus.

FIG. 2. Antisense HSV-1 LAT probes hybridize to human nodose ganglia. (A and B) HSV-1 latently infected murine sensory ganglia. Both antisense LAT probes hybridized in a characteristic nucleus-localized pattern. These tissues probed with sense probes and uninfected ganglia probed with antisense probes failed to hybridize (not shown). (C) Human nodose ganglia from a seronegative child probed with the antisense LAT 1 probe [*BstE* (T3)]. The LAT 2 antisense probe [*Hpa-Sal* (T3)] also did not hybridize to these tissues (not shown). (D and E and F and G) Adjacent 7-μm-thick tissue sections of human nodose ganglia from subject 9 showing that the nuclei of the same neurons specifically hybridized to both of the antisense LAT probes.

TABLE 1. Latent HSV-1 gene expression in human nodose ganglia

Subject	Age (yr)	Sex	Race	Cause of death	No. of LAT-positive neurons/total no. of neurons (%)		
					Side A	Side B	% Positive for A + B combined
5	84	Female	White	Pneumonia	14/7,281 (0.2)	28/5,762 (0.5)	0.3
7	62	Male	Black	Hepatocarcinoma	7/1,147 (0.6)	4/3,710 (0.1)	0.2
8	70	Female	White	Cardiovascular accident	37/6,910 (0.5)	16/6,002 (0.3)	0.4
9	67	Male	White	Myocardial infarction	70/5,475 (1.3)	81/3,939 (2.1)	1.6
10	74	Female	White	Cardiovascular accident	12/11,281 (0.1)	8/8,670 (0.1)	0.1
12	85	Male	White	Sepsis	0/10,131	0/1,366 ^a	0.0
15	81	Female	White	Myocardial infarction	0/5,853	0/6,073	0.0
17	80	Female	White	Multiple infarction dementia	2/10,545 (0.02)	0/8,532	0.01
19	72	Female	White	Respiratory arrest, sepsis	24/7,424 (0.3)	44/7,249 (0.6)	0.5
21	63	Male	White	Cardiomyopathy	72/6,738 (1.1)	NA ^b	1.1
22	48	Male	Black	Adenocarcinoma	32/9,890 (0.3)	3/9,849 (0.03)	0.2

^a Tissue sections were damaged.^b NA, not available.

HSV-1 proteins. HSV-1 produces 70 to 80 proteins during acute infection (23). In latently infected neurons, however, no virus-specific proteins are made. To further exclude the possibility that we were detecting an acute viral infection, human nodose ganglia were examined for the expression of viral proteins by indirect immunohistochemistry with polyclonal HSV-1 antiserum. We have shown that this is a sensitive method of detecting acutely infected neurons (17). When this technique was applied to tissue sections adjacent to those hybridized for LAT expression, we found that ganglion neurons which had probed positive for the HSV-1 LATs and their surrounding cells were devoid of HSV-1 proteins (Fig. 4). LAT transcripts can also be detected during acute HSV-1 infection, but they are located in the cytoplasm rather than in the nuclei of infected cells (17, 26). In the human tissues described here, the absence of viral proteins and the lack of hybridization for *ICP0* transcripts, together with nucleus-localized LAT hybridization, are indicative of latent rather than acute or reactivated neuronal HSV-1 infection.

The vagus nerve has two associated sensory ganglia, both located in the area of the jugular foramen. On the basis of animal studies, these ganglia appear to be viscerotopically organized such that the superior or jugular ganglion predominantly innervates the upper airway and hypopharynx, whereas neurons in the inferior or nodose ganglion project to the deep thoracic (esophagus, heart, and lungs) and abdominal viscera (2, 30). Neurons in the caudal pole of the nodose ganglion project to the gastrointestinal tract (2). We show here that latent HSV-1 infection frequently occurs in nodose ganglion neurons of elderly humans. Latent infection in these ganglia must reflect primary acquisition of the virus across either the gastrointestinal or lower respiratory tract.

It is known that during primary herpetic gingivostomatitis in children, HSV-1 persists in the saliva and is shed rectally for

FIG. 3. Human nodose ganglion hybridization is specific for antisense LAT probes. (A and B) Adjacent 7-μm-thick tissue sections of human nodose ganglion from subject 19. Only the antisense (T3) LAT 1 probe hybridized; the arrow in panel B (T7, sense probe) points to the same neuron shown in panel A hybridizing to the LAT 1 probe. (C and D) Adjacent sections of human nodose ganglion from subject 9 showing that only the antisense (T3) LAT 2 probe hybridized; the arrow in panel D (sense probe) points to the same neuron shown hybridizing in panel C. Under the hybridization conditions used, *ICP0* transcripts were not detected in these ganglia [*Hpa-Sal* (T7) probe] (D).

FIG. 4. HSV-1 proteins are not detected in LAT-positive neurons or surrounding satellite cells of human nodose ganglia. (A) Mouse sensory ganglion 5 days after acute HSV-1 inoculation. Infected sensory neurons and surrounding satellite cells are shown by immunohistochemistry (dark staining). (B and C) Nodose ganglion from subject 9 (7-μm-thick sections). Two neurons which hybridized with the antisense LAT 1 probe in panel B are shown on an adjacent section in panel C not to contain HSV-1 antigens. (D and E) High-power view of a LAT-positive neuron from subject 19 (D), shown on an adjacent section to be negative for HSV-1 proteins by immunohistochemistry (E).

weeks, potentially exposing nerves in the human gastrointestinal mucosa to infectious virus for a prolonged period of time (5). Furthermore, we have shown, using an animal model, that after a single intraluminal esophageal inoculation, HSV-1 can readily establish latency in the nodose ganglia (16) and can spread through the enteric nerves of mice (15, 18). These data and the fact that HSV-1 has been isolated from other human ganglia which innervate the stomach and small intestine (celiac ganglia) (27) lead us to conclude that HSV-1 latency in human nodose ganglia, particularly in the caudal pole of the ganglion, most likely results from the infection of gastrointestinal mucosal nerves caused by swallowing virus.

Sensory fields throughout the gastrointestinal surface are represented by neurons in the nodose ganglia. In the subjects studied here, the high prevalence of latency and preponderance of latent neurons within infected ganglia indicate either that nodose ganglion latency readily occurs during primary oral infection or that over time, latent HSV-1 genomes are regularly acquired from the gastrointestinal lumen after symptomatic or asymptomatic oral-labial reactivation. Seventy to 90% of adults are seropositive for HSV (7, 25). The frequency of recurrent oral-labial disease is approximately 33% (12), and it is estimated that asymptomatic trigeminal ganglion reactivation and oral excretion can be detected in 1 to 5% of normal adults and children at any given time (11). Virus reactivation in latently infected human nodose ganglia may occur with a similar frequency. If this is true, it is reasonable then to conclude that a number of recurrent functional or inflammatory gastrointestinal disorders may directly or indirectly be attributed to enteric HSV-1 infection.

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