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Patterns of gene expression and sites of latency in human nerve ganglia are different for varicella-zoster and herpes simplex viruses

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ABSTRACT **The cellular localization and viral transcription patterns of acute and latent varicella-zoster virus (VZV) infections of human sensory nerve ganglia were studied by *in situ* hybridization and compared with those of latent herpes simplex virus (HSV) infection. Trigeminal and dorsal root ganglia obtained at autopsy were hybridized with ^{35}S-labeled single-stranded RNA probes homologous to VZV or HSV fragments. We have reported that HSV persists in human sensory neurons and expresses only one family of transcripts that overlap extensively with, but are opposite in polarity to, the mRNA encoding the immediate early protein termed infected cell protein 0 (ICP0). In the present study we find that latent VZV infection involves nonneuronal cells, and multiple, but not all, VZV genes are transcribed. In contrast, during varicella both neuronal and nonneuronal cells are infected, with all regions of the VZV genome analyzed being expressed. Thus, the patterns of gene expression and cellular locations of VZV and HSV infections of human ganglia differ. The differences may underlie clinical features that distinguish these infections.**

The neurotropic human herpesviruses, herpes simplex viruses (HSV) types 1 and 2 and varicella-zoster virus (VZV), cause similar, though clinically distinguishable, infections. Varicella is a disseminated infection while HSV infections are typically localized ones. During primary infections, both viruses establish latency in sensory ganglia from where they reactivate to produce recurrent disease (1, 2). Whereas HSV infections recur numerous times, VZV infections rarely recur more than once. The mild discomfort accompanying localized recurrences of HSV infections contrasts sharply with the severe pain of a recurrence of VZV infection (zoster). In addition, zoster gradually spreads to encompass much of a single cutaneous dermatome, while the lesions of recurrent HSV are few, and limited in distribution. These differential features of primary and recurrent infections with HSV and VZV suggest that the nature of their acute and latent infections in sensory ganglia and their mechanisms of reactivation could differ.

A variety of techniques can now be used to define the comparative biology of these infections. It is already evident that HSV persists within sensory neuronal cells and remains transcriptionally active. Recent studies of latently infected trigeminal ganglia from mice, rabbits, and humans established that virus-specific transcription is limited to the long repeat segments of the HSV-1 genome (3–10). Latency-associated RNA is found almost exclusively within ganglion cells when probed by *in situ* hybridization (see Fig. 2*a*) (3, 5–10). By Northern RNA gel blot hybridization, the latency-associated RNA consists of two or three colinear transcripts (7, 9, 10). These transcripts overlap extensively with, but are opposite in polarity to, the transcript encoding the HSV-1 immediate early protein ICP0 (infected-cell protein 0).

The status of VZV during latency is less clear. Within the past few years VZV DNA and RNA were detected in latently infected human sensory ganglia. Up to 1 copy per cell of VZV DNA was demonstrated by Southern hybridization of trigeminal ganglia from 3 of 9 individuals (11). Our own efforts to detect VZV DNA by Southern hybridization studies of 23 adult human ganglia (18 trigeminal, 4 thoracic, and 1 lumbar ganglion from 22 subjects) were unsuccessful (K.D.C., unpublished observation). Detection of VZV RNA in human sensory nerve ganglia has also been reported. The conclusion of those studies, involving *in situ* hybridization, is that latent VZV resides in ganglion neuronal cells (12, 13).

To better characterize the cellular location and nature of VZV transcription in latently infected human sensory nerve ganglia and to contrast it with that of acute varicella and of latent HSV-1 infection, we conducted the present study. *In situ* hybridizations were performed by using ^{35}S-labeled single-stranded RNA probes. The patterns of VZV and HSV-1 gene expression and their cellular localizations were found to differ.

MATERIALS AND METHODS

Tissues and Their Preparation. Trigeminal and dorsal root ganglia were removed at autopsy and dissected free of surrounding tissue. The ganglia were frozen in dry ice or fixed in 10% (vol/vol) formalin and then embeded in OCT compound (Miles Scientific, Chicago, IL) or paraffin, respectively. Sections of 6–8 μm were placed onto slides that had been acid-washed and treated with 3-aminopropyltriethoxysilane (14). After sectioning, the frozen tissues were fixed in a periodate/lysine/paraformaldehyde/glutaraldehyde solution (15) and stored at −20°C. The paraffin-embedded tissue sections were dewaxed immediately prior to use.

RNA Probes. Plasmid libraries of VZV and HSV-1 DNA restriction fragments were recloned into pGEM vectors (Promega Biotec). The Epstein–Barr virus *Bam*HI fragment W in pSP64 was a gift of E. Kieff (Harvard University). Unidirectional RNA probes labeled with uridine 5′-[α-[^{35}S]-thio]triphosphate (DuPont/New England Nuclear) to specific activities of 2–5 × 10^8 cpm/μg were synthesized from these templates and alkaline-hydrolyzed to reduce the probe size, as described (6).

***In Situ* Hybridization and Autoradiography.** The hybridization procedures have been described (6, 15, 16). Briefly, the fixed sectioned tissues were digested with proteinase K (Sigma) and acetylated. Some slides were treated with RNases A (Sigma) and T1 (Sigma), or DNase I (Sigma). Nuclease-treated slides were post-fixed in paraformaldehyde and dehydrated with ethanol. Denaturation of sample DNA

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Abbreviations: VZV, varicella-zoster virus; HSV, herpes simplex virus; ICP0, infected cell protein 0; ORF, open reading frame.

was achieved by heating in 95% (vol/vol) formamide/0.1× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) to 65°C for 10 min followed by 0.1× SSC at 4°C for 5 min. Hybridization was performed overnight in the dark at 46–60°C depending on the G+C percent content of the probe. Slides were washed at 60–65°C, dipped in NTB3 emulsion (Eastman Kodak), and exposed for 3–33 days at 4°C, as indicated. Slides were developed in D-19 (Eastman Kodak), fixed in 30% (wt/vol) sodium thiosulfate, and stained with hematoxylin and eosin. A minimum of four sections of every ganglion were evaluated.

Serologies. Antibody titers for VZV were determined for serum collected at autopsy by heart puncture by using the indirect immunofluorescence antibody assay. Sera that were negative by that assay were rechecked with the more sensitive fluorescent anti-membrane antibody assay, courtesy of A. Gershon and S. Steinberg (Columbia University) (17).

RESULTS

Trigeminal ganglia were obtained at autopsy from individuals within 4–48 hr of death (median, 19 hr). None of the cadavers whose tissues were used in latency studies had evidence of active HSV or VZV infection or were known to be immunologically compromised. Most died of substance abuse, gunshot wounds, vehicular accidents, or sudden infant death syndrome. The tissues were fixed, embedded, sectioned, and hybridized with individual or pooled ^{35}S-labeled single-stranded RNA probes transcribed from HSV-1 or VZV DNA fragments that had been cloned into vectors containing bacteriophage SP6, T3, or T7 RNA polymerase promoters (Fig. 1). Table 1 summarizes the results of these hybridizations.

As reported (6), hybridization signals associated with latent HSV transcripts in human trigeminal ganglia were found in 0.2–4.3% of neuronal cells in ganglia from 14 of 24 individuals (Fig. 2*a*) (6). Hybridization of trigeminal ganglia obtained from an additional 37 autopsies brings the total to 34 of 61 individuals showing evidence of latent HSV infection. The ganglia from all 5 infants studied (Table 1) as well as those from all adults lacking HSV antibodies also lacked detectable HSV RNA (6). The only probes yielding hybridization signals were from the regions adjoining the ICP0 gene, and the transcripts they detected were from the strand opposite to

Table 1. Results of *in situ* hybridization for VZV and HSV in latently infected human trigeminal ganglia

Specimen	Age	VZV serology	VZV signal	HSV signal
1	3 weeks	*	−	ND
2	6 weeks	−	−	ND
3	3 months	*	−	ND
4	3 months	*	−	−
5	3 months	*	−	−
6	3 months	*	−	−
7	4 months	*	−	−
8	7 months	*	−	ND
9	7 months	*	−	−
10	23 years	+	+	+
11	28 years	+	−	+
12	37 years	+	−	+
13	43 years	+	+	+
14	46 years	+	−	+
15	47 years	+	+	−
16	51 years	+	+	+
17	63 years	+	−	−
18	9 years	NA	+	+
19	20 years	NA	+	+
20	26 years	NA	+	+
21	28 years	NA	+	+
22	30 years	NA	+	+
23	34 years	NA	+	−
24	35 years	NA	−	−
25	36 years	NA	−	+
26	38 years	NA	+	+
27	47 years	NA	+	+
28	53 years	NA	+	−
29	67 years	NA	+	+
30	74 years	NA	+	−

ND, not done; NA, serum not available; *, no history of varicella, but a low positive antibody titer was detected that is compatible with passive transfer of maternal antibody; −, negative; +, positive.

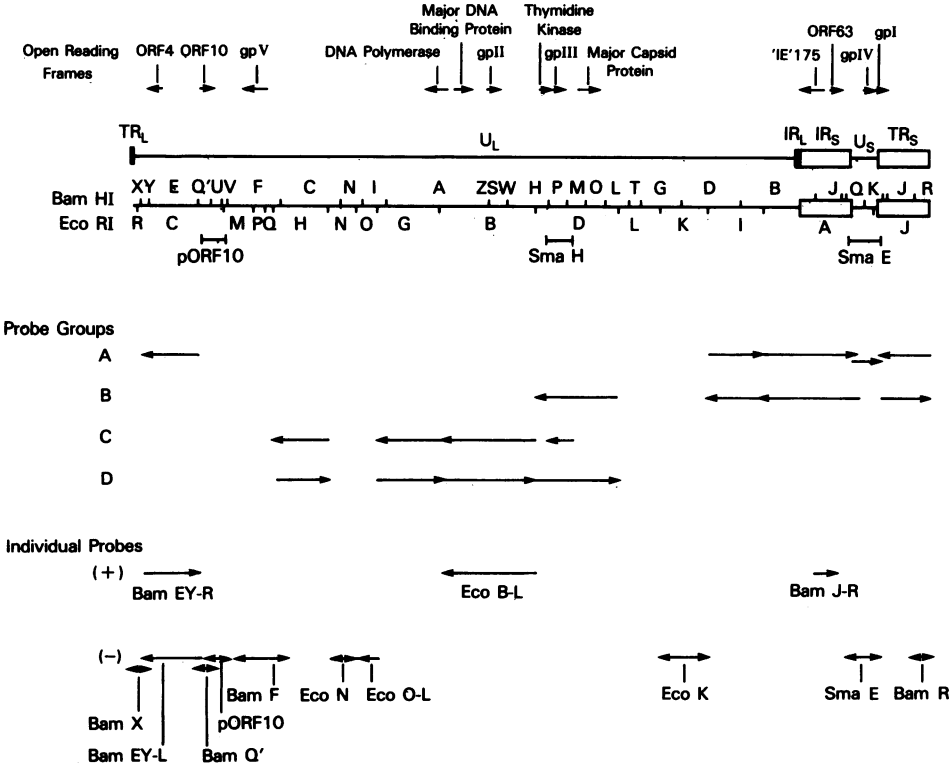

FIG. 1. Map of the VZV genome showing long and short unique regions (U_L and U_S, respectively) and repeats flanking the U_S (IR_S and TR_S) and U_L (IR_L and TR_L) as well as relevant restriction sites. Selected gene products and open reading frames (ORFs) of the genome are shown (18). ORF4 and IE175 encode the VZV equivalents of the HSV-1 immediate early infected-cell proteins 27 and 4, respectively. ORF 10 encodes the equivalent of the HSV-1 α-trans-inducing factor. RNA probes were transcribed in either direction (as indicated by arrowheads) from templates spanning the VZV genome and were divided into four pool groups A–D. Hybridization signals were detected with each of the probe groups. Individual RNA probes that detected VZV latency transcription (+ probes) include the rightward (R) products from plasmids pGVBamHI-EY and pGVBamHI-J (probes Bam-EY-R and BamJ-R, respectively), as well as the leftward (L) product from pGVEcoRI-B (EcoB-L) (19). Probes not detecting VZV RNA during latency are shown at the bottom of the figure (− probes).

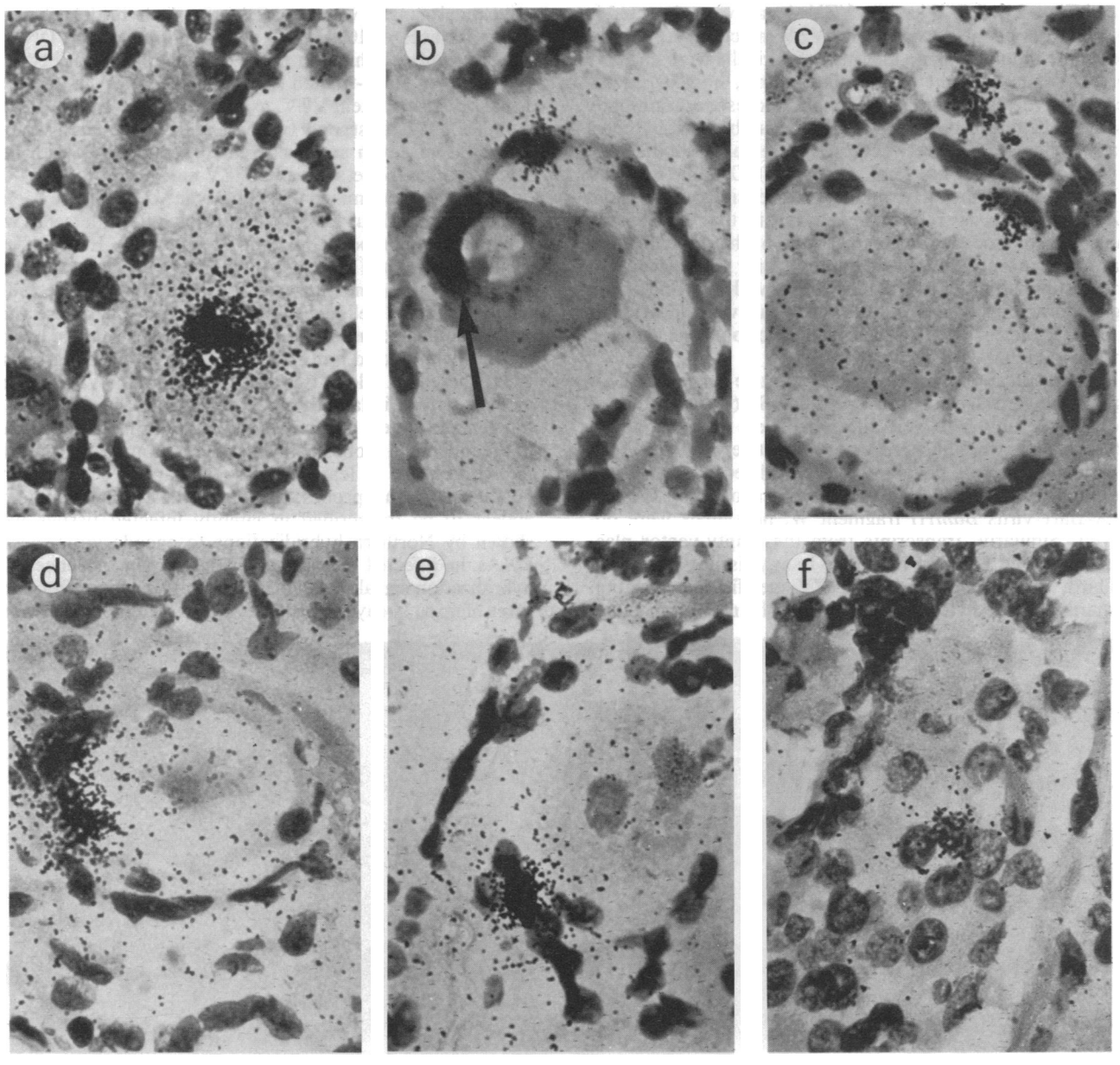

FIG. 2. *In situ* hybridizations of latently infected human trigeminal ganglia for HSV and VZV RNA. The large neuronal cells are surrounded by smaller satellite cells and other nonneuronal cells. The signal for latency-associated transcripts of HSV-1 is demonstrated over a ganglion cell nucleus by hybridization with an RNA probe synthesized in the leftward direction from the 2866-base-pair *Sph* I subfragment of *Bam*HI fragment E (10). Latency-associated VZV RNAs in satellite cells adjacent to negative neuronal cells are shown in *b–e* and in a fibroblast-like cell is in *f*. Probes used on these sections were from the groups of pooled probes (see Fig. 1). The arrowhead in *b* points to an artifactual signal overlying a large perinuclear deposit of lipofuscin pigment. All slides were exposed for 4–7 days. (×570.)

that encoding the ICP0 message. RNase treatment before hybridization markedly reduced the intensity of the signal; DNase treatment did not. Hybridization with probes from all other portions of the HSV genome were negative. In nearly all samples, the hybridization signals for HSV RNA were localized to the neuronal cell nuclei (Fig. 2*a*). The distribution of silver grains in the occasional very strong hybridization suggests that in some cells the HSV latency-associated RNA could be in the cytoplasm. In a small fraction of tissues (4 of 60 positive sections), isolated nonneuronal cell signals were also observed, consistent with an observation made in the mouse model of HSV latency (20).

HSV-specific hybridization signals were readily distinguished from silver grains overlying neuronal cell lipofuscin pigment (21). Lipofuscin appears as granular yellow–brown perinuclear aggregates within the cytoplasm, though occasionally it may be distributed diffusely. This pigment may lead to nonspecific autoradiographic signals (an example of which is shown in Fig. 2*b*).

In contrast to the findings with latent HSV infection, hybridizations for latent VZV with individual or pooled probes representing nearly the entire VZV genome (Fig. 1) yielded signals exclusively over a small fraction (0.01–0.15%) of the nonneuronal cells in ganglia from 15 of 30 people (Fig. 2 *b–f*). Such cells include satellite cells, fibroblasts, and endothelial cells. Because of their proximity to neuronal cells, most of the signals were felt to overlie satellite cells. These cells are of ectodermal origin and appear to be the ganglion equivalent of the Schwann cell, though their function is unknown. No signals other than ones that are clearly attributable to lipofuscin (Fig. 2*b*) were ever detected over ganglion cells, even with exposures of up to 33 days.

A variety of studies were performed to validate the specificity of the hybridization signal for latent VZV (Table

1 and Figs. 2 and 3). First, no VZV signals were detected in tissues from people lacking prior VZV infection. All ganglia from nine infants with no histories of varicella were negative for VZV RNA. Eight of the nine infants, however, were seropositive by the indirect immunofluorescence antibody assay or the fluorescent anti-membrane antibody assay, most likely indicating passively acquired maternal antibodies that remain detectable for several months (17). Ganglia from four of the eight seropositive adults yielded signals for latent VZV. That any were negative suggests that all sensory ganglia are not seeded during varicella or that this infection may involve only limited portions of any single ganglion. These suppositions are also consistent with the findings in eight thoracic ganglia from four adult cadavers (individuals 11, 24, 25, and 26; listed in Table 1) in which only one ganglion (from individual 24) was positive.

Second, of 15 trigeminal ganglia positive for latent VZV signals, 11 were also positive for latent HSV (Table 1), though the cell types involved were different. There were four adults whose ganglia were positive for HSV, but negative for VZV. When 11 trigeminal ganglia were hybridized with an RNA probe capable of detecting transcripts encoded by the Epstein–Barr virus *Bam*HI fragment W, no signals were observed. Similarly, transcripts from the empty-vector plasmids employed failed to hybridize to any tissue.

Third, we verified that our VZV-specific hybridization primarily detected viral RNA. Sections of nine VZV-positive trigeminal ganglia were treated prior to hybridization with either RNases A (100 μg/ml) and T1 (10 units/ml), DNase I (50 units/ml), all three enzymes, or none, as described (16). Six of seven mock-treated sections yielded typical, nonneuronal signals for latent VZV, as did six of nine DNase-treated samples. In contrast, only two of nine RNase-treated samples and none of seven sections treated sequentially with both RNases and DNase showed a VZV signal.

Fourth, we determined that only some VZV genes are active during latency. Thus far, transcripts have been detected with unidirectional probes from *Bam*HI fragments EY and J, as well as from *Eco*RI fragment B (shown in Fig. 1). All of these probes are complementary to VZV transcripts detected in acutely infected cells (see Fig. 1) (18, 19, 22). These three probes could account for positive signals observed in hybridizations with pooled probe groups A and C, but not groups B or D. Therefore, at least two other regions of the VZV genome are transcribed in latently infected ganglia. Many regions of the genome appear not to be expressed during latency. Each of the individual probes (shown at the bottom of Fig. 1) failed to hybridize to at least six tissues that were consistently positive with other probes. Regions of the genome that do not appear to be transcribed in latently infected tissues were shown by Northern hybridizations to encode virus-specific RNAs in infected fibroblasts (19, 22). The absence of a hybridization signal in ganglia from these regions after exposures of up to 14 days is further evidence that the viral genome

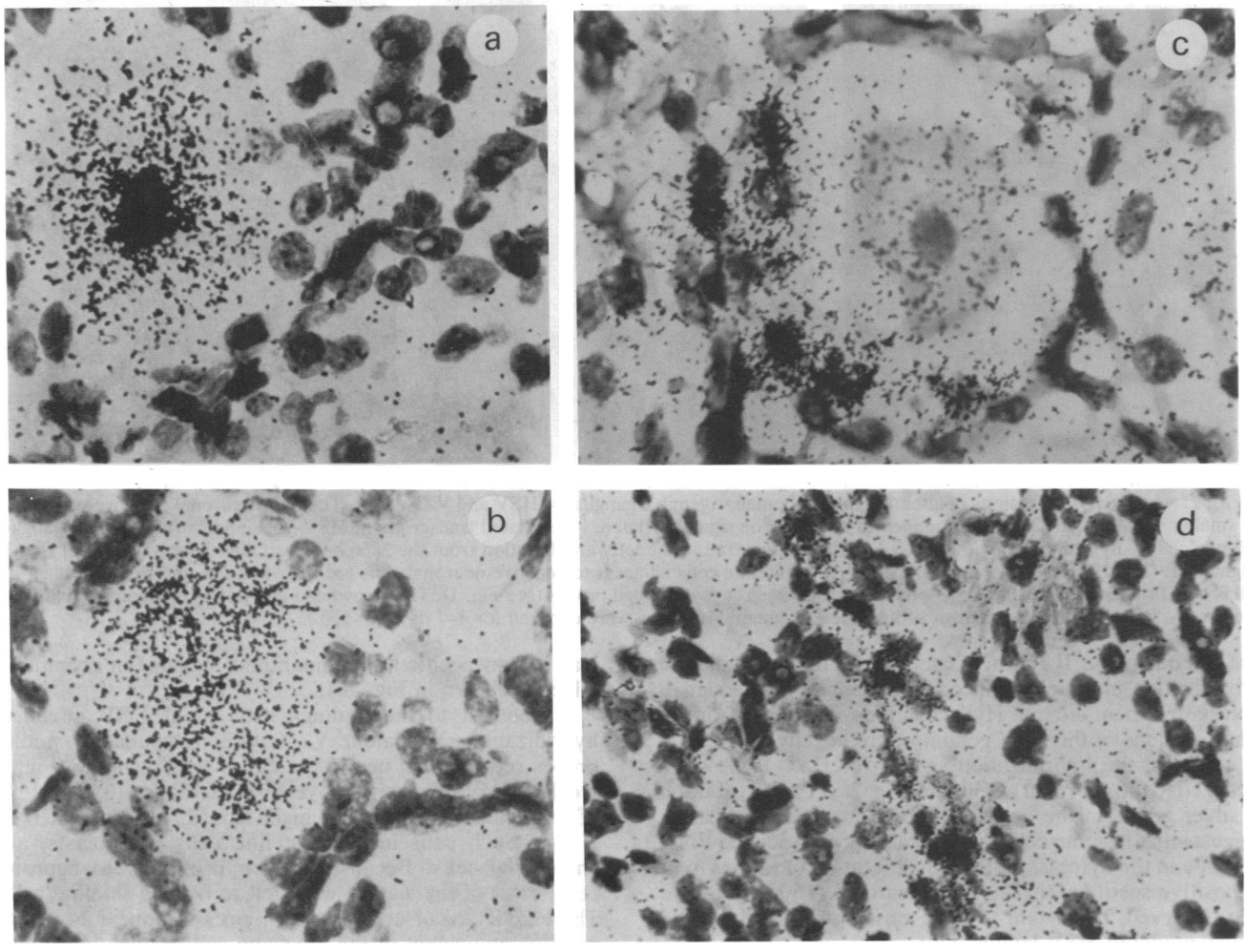

FIG. 3. *In situ* hybridizations of human trigeminal ganglia recovered during acute VZV infection. (*a* and *b*) Nuclear and cytoplasmic signals over ganglion cells. These sections were probed with RNAs transcribed from a pool of VZV fragments (*a*) and from VZV *Sma* I fragment E (leftward probe shown in Fig. 1) (*b*). A cluster of infected satellite cells without adjacent ganglion cell involvement is shown in *c*. Infection of other nonneuronal cells (fibroblasts, endothelial cells, or Schwann cells) are shown in *d*. The probe for *c* and *d* was also *Sma* E-L (as in *b*). All slides were exposed for 4–7 days. (×475.)

is not being detected and that the infections observed are abortive rather than fully productive.

We were also able to study the trigeminal ganglia recovered from one additional autopsy, that of an 8-year-old child with cerebral palsy who died on the sixth day of an active varicella infection. This gave us the opportunity to compare latent infection with primary varicella infection of sensory nerve ganglia. Approximately 0.6% of this child's ganglion neuronal cells showed nuclear or nuclear and cytoplasmic signals (Fig. 3 *a* and *b*). Approximately 0.3% of nonneuronal cells were also positive (Fig. 3 *c* and *d*), but in only one instance were such cells adjacent to an infected ganglion cell. Most of the infected nonneuronal cells were clustered as shown in Fig. 3 *c* and *d*. The distribution of the VZV infection within this child's ganglia is similar to that reported (23) by immunofluorescence and electron microscopy of thoracic ganglia from a patient with fatal varicella.

DISCUSSION

Latent HSV and VZV infections of human ganglia are different. HSV resides almost exclusively within neuronal cells and expresses only one family of abundant transcripts that overlap with the ICP0 message. In contrast, VZV establishes latency in satellite and perhaps other nonneuronal cells and leaves no apparent trace of the active ganglion cell involvement observed during varicella. Furthermore, VZV latency involves transcription from several regions of its genome.

These data conflict with those reported with regard to the cellular location of VZV latency (12, 13). Those observations of VZV hybridization signals overlying ganglion cells may be explained by differences in the hybridization techniques and/or the existence of aggregates of lipofuscin pigment within such cells (21). The large number of samples tested, the high sensitivity and resolution afforded by the present *in situ* technique, and the numerous studies performed to validate the hybridization results, however, strongly favor our findings. We compared the findings in latent VZV infection with those of acute varicella and latent HSV-1 infections. The specificity of the hybridization results were verified serologically in that tissues from individuals with no evidence of prior varicella showed no VZV hybridization signals. Nuclease treatments showed the VZV signals to indicate primarily the presence of virus-specific RNAs in the tissues. Finally, regions of the genome that are actively expressed in productive replicative cycles, but that are quiescent in latency, were identified.

The present observations, together with those made by others in studies of primary HSV and VZV infections, may help explain some of the differences in the patterns of reactivation of each virus. Our hybridization findings and the results of electron microscopic studies of ganglia during varicella indicate that VZV replicates efficiently in both neuronal and nonneuronal cells (23). VZV remains latent and can reactivate in the context of a nonneuronal cell that can support its replication and facilitate its spread to adjacent susceptible neuronal and nonneuronal cells. Eventually, large portions of a ganglion are destroyed by the spreading infections (24, 25). In the process, multiple sensory nerves become infected and convey the virus to all quadrants of the cutaneous dermatome. The inflammation and necrosis can incite a severe and chronic neuropathy. HSV, however, reactivates within a neuronal cell that is surrounded by satellite cells which, according to electron microscopic studies of acutely infected mouse ganglia, are less capable of supporting its productive replication (26–29). Virus spreads readily to the skin but it does not spread efficiently within the ganglion so that cutaneous involvement and neuropathy are limited.

The relative infrequency of VZV reactivation may reflect its nonneuronal site of latency, rendering it less subject to the neural triggers that provoke HSV reactivation. There also may be a variety of physical, metabolic, or immune obstacles to the spread of VZV from its site of persistence, to the neural "conduit" through which it is transported to the skin. The relative infrequency of recurrent VZV infections could also relate to the mechanisms by which VZV remains latent. VZV appears to lack a gene equivalent to that for HSV ICP0 (18). If this gene or the overlapping latency transcript regulate the latent state of HSV, then a different mechanism must have evolved to ensure VZV latency. With several genes actively expressed during latency, VZV may exploit multiple, and perhaps more potent, regulatory strategies to sustain its latent state. A fuller characterization of the latency-associated transcripts of VZV will indicate whether they are all identical to those transcribed during acute infection, or as with HSV, some are "anti-sense" to such transcripts.

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