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Varicella-Zoster Virus Infection of Adult Rat Sensory Neurons In Vitro

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We report here an in vitro model of neuronal infection by varicella-zoster virus (VZV). Such a model has been achieved by using dissociated adult rat dorsal root ganglia cells infected by cocultivation with VZV-infected MRC5 cells or with cell-free virus. Indirect VZV immunolabeling, in situ hybridization, and neuron-specific immunolabeling demonstrated that VZV infection occurred selectively in neurons. VZV-specific immunolabeling detected a few neurons 1 or 2 days postinfection but not later. Genome detection using cloned VZV DNA probes revealed a hybridization signal primarily with RNA. Within 1 to 6 days postinfection, a progressive increase of VZV-specific hybridization was observed in up to 50% of the neurons. RNAs corresponding to immediate-early, early, and late genes were found, and transcripts of immediate-early gene 63 were particularly abundant.

Varicella-zoster virus (VZV) is a human herpesvirus which causes a primary infection in childhood, becomes latent, presumably in dorsal root ganglia (DRG), and is reactivated many years later to produce shingles in adults (14). VZV has been localized by genomic hybridization techniques in human ganglia which have not been recently exposed to VZV (12, 15). VZV-specific proteins have been detected in explanted human ganglia and may be related to genes 62 and 63. Moreover, in situ hybridization revealed the presence of gene 63 RNA in the same ganglia, suggesting that immediate-early (IE) genes 62 and 63 expressed during latency may play a role in establishing and maintaining VZV latency in humans (21).

Knowledge concerning the molecular aspects of VZV infection is rudimentary in comparison with that relating to other human herpesviruses. One major reason is that, even though VZV is a highly productive virus in skin cells during clinical infection, it remains predominantly cell associated throughout its replication cycle in vitro. Molecular cloning and physical mapping of the VZV genome (9, 20) and its complete DNA sequence (7) have provided important information on the size of the genome and gene organization. There are several polypeptide homologs between herpes simplex virus type 1 (HSV-1) and VZV, and from these, three putative IE genes (ORF4, ORF62, and ORF63) have been identified (7, 11). In productive systems, transcription mapping of the VZV genome revealed 58 unique transcripts ranging in size from 0.8 to 6.5 kilobases (kb) and encoded by sequences spanning the entire genome (16).

The first study of VZV-neuron interaction reported the acute infection of human fetal DRG cells which resulted in the development of virus-specific cytopathic effect, viral antigen expression, and production of virus particles (22). Since the peripheral nervous system is adult when the initial infection occurs in childhood and because virus reactivation happens in adults, leading to shingles, we have chosen adult rat ganglia neurons in dissociated cultures to study VZV infection and gene expression in nerve cells.

Two strains of VZV were used in these studies; they were isolated by sterile aspiration of vesicular fluids of patients during varicella infection. Restriction analysis of the purified DNAs shows the typical pattern of VZV strain Ellen. Infected MRC5 (human embryonic lung) cells and cell-free virus were used as inocula. Cell-free virus was obtained by ultrasonication of infected MRC5 cells in PSGC medium (5% sucrose, 0.1% sodium glutamate, 10% fetal calf serum in phosphate-buffered saline [PBS]) and clarified by centrifugation at 5,000 × *g* (10 min, 4°C) (13). Stocks were stored at −80°C.

Neuronal cultures were prepared by using DRG dissected from 3- to 6-month-old Wistar rats essentially as described in prior characterization of the DRG cultures (P. Delrée, P. Leprince, J. Schoenen, and G. Moonen, J. Neurosci. Res., in press). Notice however that the last purification step of the procedure described by Delrée et al. (in press) was omitted in the present study so that mixed neuronal and nonneuronal cell populations were obtained. Seeding density was 800 to 1,200 neurons per cover slip, and cultures contained 10% neurons.

At 24 h after being seeded in culture, adult rat dorsal ganglia neurons were infected with VZV by overlaying cover slips with a trypsinized suspension of an equivalent surface area of VZV-infected MRC5 cells or with cell-free virus at a multiplicity of infection of 0.1. After 18 h of incubation, the inoculum was removed and washed with culture medium, and cells were maintained at 37°C in Dulbecco modified essential medium-fetal calf serum (5%). After various incubation times (1 to 10 days), the cover slips were washed in PBS and fixed in acetone for immunostaining or in paraformaldehyde (4%) for in situ hybridization.

VZV DNA was purified from nucleocapsids (20), and VZV DNA fragments generated by digestion with *Bam*HI or *Eco*RI were cloned into pBR322 and pUC19, respectively, and amplified in *Escherichia coli* HB101. Cloned DNA fragments were labeled in vitro by nick translation (18) or random priming (10) with biotin 11-dUTP (Bethesda Research Laboratories, Inc.) or with [^{35}S]dCTP (Amersham Corp.). The incorporation of biotin was checked by dotting the probes on nitrocellulose, exposing them to an avidin-

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peroxidase complex, and revealing them with H_2O_2 (0.1%)–3,3′ diaminobenzidine-tetrachlorhydrate (0.5 mg/ml).

Infected and control cells used for in situ hybridization studies (2, 4) were treated in 0.2 N HCl (10 min)–0.1% Triton X-100 (2 min) followed by 15 min at 37°C in 20 mM Tris hydrochloride (pH 7.4) containing 1 μg of proteinase K per ml. Samples treated or untreated with RNase were given a 15-min postfixation in 4% paraformaldehyde in PBS and washed in PBS for 5 min. The hybridization mixture contained 50% (vol/vol) deionized formamide, 10% (wt/vol) dextran sulfate, 250 μg of sonicated carrier DNA (herring sperm) per ml, 2× SSC (1× is 0.15 M NaCl, 0.015 M sodium citrate), and 4 μg of denatured probe DNA per ml.

We have combined in situ hybridization and immunolabeling to detect VZV nucleic acids and specific neuron proteins in the same cells. Infected cells were fixed with 0.5% formaldehyde (10 min at room temperature) followed by a 5-min incubation in 70% ethanol. The neurons were labeled with an antineurofilament monoclonal antibody that was revealed with peroxidase-labeled rabbit anti-mouse immunoglobulin G (IgG) amplified with a peroxidase rabbit antiperoxidase complex. After being stained with DAB, the same cells were immediately treated for in situ hybridization with ^{35}S-labeled probes. After proteolytic treatment (see above), acetylated cells were postfixed in paraformaldehyde (4% in PBS), dehydrated in graded ethanol solutions, and prehybridized at 37°C for 3 h. Then, 10^6 cpm of the ^{35}S-labeled probe was added to the solution, and hybridization was for 18 h at 37°C. Extensive washing was carried out at 65°C in 0.2× SSC.

The structure of the large sensory neurons in infected cultures appeared to be rather well preserved, and infected cultures survived for at least 10 days. Staining of the neurons with an antineurofilament monoclonal antibody demonstrated the presence of neurofilaments in cell bodies and processes of the neuronal cells (Fig. 1a). No differences were observed between infected and noninfected control neurons, and the number of cells remained stable, indicating that the cells survived the infection. During the 1 to 10 days of the adult rat DRG cultivation, the virus was not released in the culture medium, as verified by medium inoculation to MRC5 cell culture. No cytopathic effect was detected, and no viral antigens appeared in MRC5 cells even after 8 days in culture with medium conditioned for various periods (up to 7 days) by infected neuronal cells. In addition, VZV antigens were not detected in the DRG culture medium by enzyme immunoassay. The virus was thus maintained in DRG cultures as cell-associated virus.

The presence of cell-associated VZV antigens was examined by indirect immunofluorescence of the surfaces of living cells with a human serum containing a high titer of anti-VZV antibodies revealed with a fluorescein-conjugated monoclonal anti-human IgG. Within 1 to 2 days after infection, only a few neurons (1%) expressed VZV antigens (Fig. 1b). At later times in the infection process, antigen expression decreased and disappeared totally by 5 days after infection. In situ hybridization experiments were done first with probes spanning the complete genome and labeled with biotin. Control experiments were done with uninfected or VZV-infected MRC5 cells (Fig. 1c). Neither a vector plasmid (M13mp19) nor a human cytomegalovirus probe, both labeled with biotin, could hybridize to these VZV-infected cells. Hybridization of HSV-infected MRC5 cells with VZV probes was negative. Examination of infected adult rat DRG cultures revealed a strong hybridization between the labeled probe and VZV nucleic acid sequences. This hybridization was restricted to neurons; nonneuronal cell (fibroblasts and Schwann cells) turned out to be free of viral nucleic acids (Fig. 1d). In most neurons, the labeling was mainly observed over the cytoplasmic area, including processes. However, hybridization reactions could also be observed over some neuronal nuclei, although with various intensity levels. Treatment of the neuron cultures with ribonuclease reduced significantly the hybridization signal, indicating that hybridization of the VZV probe was primarily with VZV mRNA. This finding suggests that the VZV DNA copy number in the neuronal nuclei is low and that active VZV replication does not take place in these cultured rat neurons.

Within 1 to 6 days after VZV infection of the adult rat DRG neuron cultures, a progressive increase of VZV-specific hybridization was observed. As early as 1 day after infection, 20% of the neurons were infected by VZV. This percentage increased regularly and reached 50% after 6 days (Fig. 2). However, by 8 to 10 days after infection, the number of infected neurons remained stable, and beyond that time, we observed enlargement and aggregation of some neurons. In order to select a preferential infection window, the DRG cultures were infected at various times (1 to 9 days) after dissociation and seeding in culture. These cultures were studied by in situ hybridization 3 days after infection. The proportion of positive neurons exhibiting a hybridization signal remained the same whatever the delay between neuronal seeding and infection by coculture with MRC5 cells. In addition, the same results in terms of efficiency of infection were obtained when the DRG cultures were infected at the time of seeding, and we can conclude that the percentage of infected neurons remained identical regardless of the time in culture before VZV infection.

Although it is easy to distinguish neurons from nonneuronal cells on the sole basis of morphological criteria, we have tried to detect simultaneously the presence of VZV nucleic acids and neuron-specific proteins in the same cells. This double labeling could be done after modifying the conditions of cell fixation and hybridization procedures to preserve the reactivity of nucleic acids and antigens. As expected, detection of neurofilaments had to be done first, since antigens are destroyed by hybridization procedures (3). When ^{35}S-labeled VZV probes were used, the cells exhibiting a hybridization signal were also labeled by antineurofilament antibodies (Fig. 3). These results demonstrated conclusively that VZV infection of DRG cultures occurred selectively in neurons.

The results obtained by immunolabeling and cytohybridization indicated that VZV infection in neurons by cocultivation was nonproductive. To segregate between an abortive and a persistent infection, we have analyzed VZV gene transcription in infected neurons and compared it with VZV or HSV-1 gene transcription in human latent neuron infection (5, 21). The VZV-specific transcripts in infected neurons were analyzed by in situ hybridization with probes specific for VZV genes that were representative of the temporal gene classes (IE, early, and late). The 4.0-kb subfragment obtained after successive restrictions of the VZV *Eco*RI A fragment with *Ssp*I (7.0 kb) and *Tth*III-I was used to detect RNA from the regions encoding IE gene 62. A 0.9-kb probe corresponding to the *Eco*RI A-*Ssp*I subfragment (4.0 kb) restricted by *Hpa*I and *Bam*HI was used to detect RNA from IE gene 63. The *Eco*RI C-*Kpn*I (3.4-kb) subfragment and the *Bam*HI H-*Pst*I (2.6-kb) subfragment were used to detect RNA from the regions encoding IE gene 4 and early gene 36 (thymidine kinase), respectively. The *Eco*RI B-*Hin*dII (2.6-kb) probe was used to detect specifically gpII RNA (a late

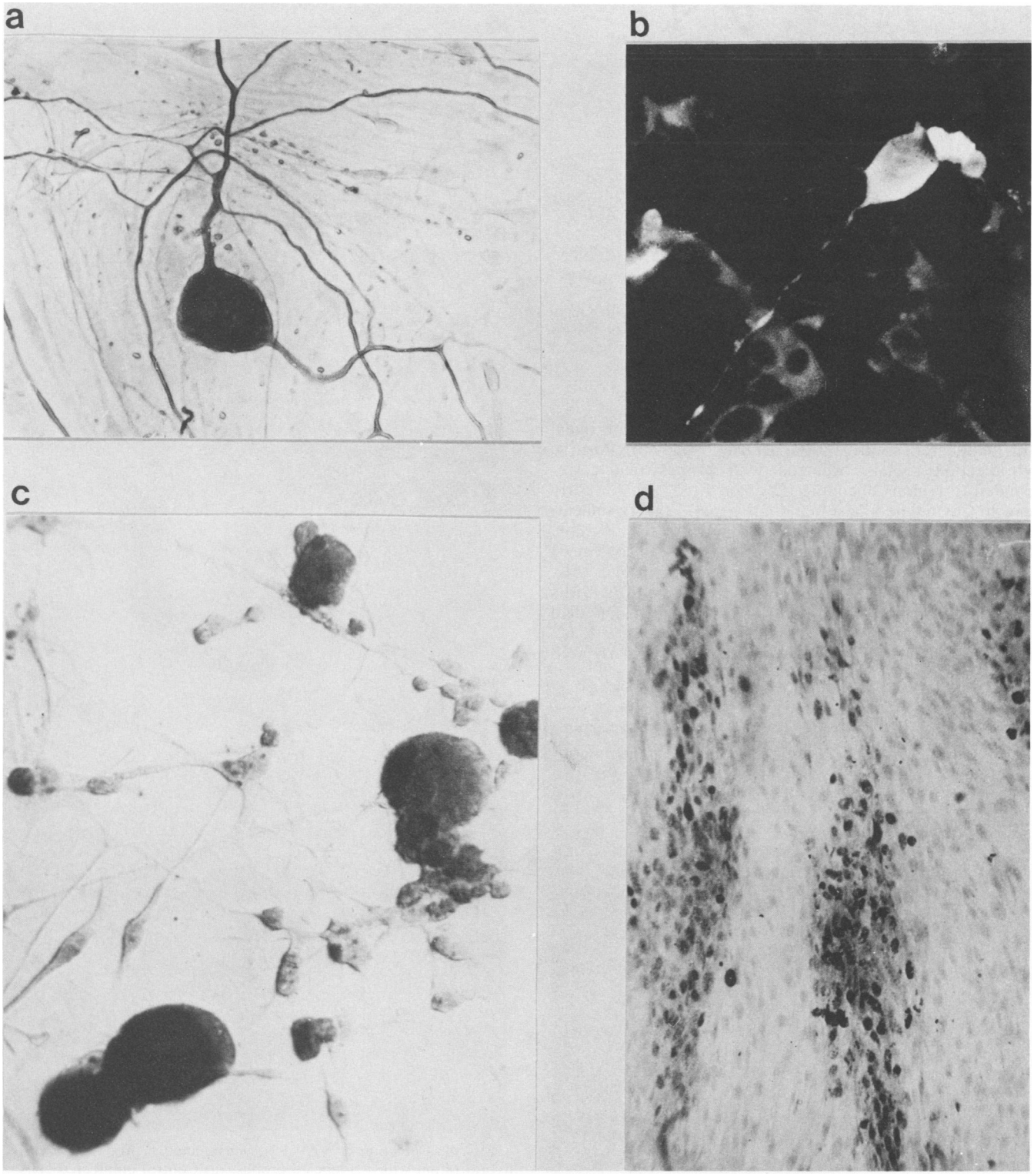

FIG. 1. Indirect immunolabeling and in situ hybridization analysis of adult rat DRG neurons after VZV infection (2 days postinfection). (a) Primary monoclonal antineurofilament followed by a peroxidase-conjugated rabbit anti-mouse IgG antibody. (b) Primary human serum rich in anti-VZV antibodies followed by a fluorescein-conjugated monoclonal anti-human IgG antibody. Magnification, ×410. (c) In situ hybridization of infected MRC5 cells probed with VZV DNA *Bam*HI fragments labeled with biotin. Magnification, ×220. (d) In situ hybridization (biotin probes) of infected adult rat DRG neurons at 2 days postinfection. Magnification, ×640. The VZV DNA probe was an equimolar mixture of recombinant VZV DNAs spanning the VZV genome, *Bam*HI A, B, C, E, H, I, J, K fragments. After being washed, biotin probes were revealed with goat anti-biotin antibody and peroxidase-labeled rabbit anti-goat IgG.

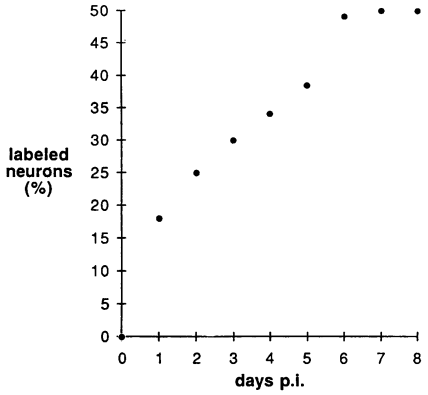

FIG. 2. Percentage of infected neurons obtained after 1 to 8 days postinfection (p.i.). This percentage was determined after in situ hybridization by counting neurons with a positive or a negative hybridization signal.

gene). None of these probes exhibited a hybridization signal with uninfected control DRG neurons (Fig. 4a). Positive hybridization signals occurred with probes that corresponded to regions encoding IE genes 4, 62, and 63, early gene 36 (thymidine kinase), and late gene 31 (gpII), indicating that these genes are transcribed in infected neurons. Hybridization with the fragment corresponding to IE gene 63 was noticeably stronger than that with the other probes (Fig. 4b and c). Similarly, neurons infected with cell-free virus showed positive hybridization signals, with individual probes corresponding to regions encoding IE genes 4, 62, and 63 and late gene 31. These results fit particularly well

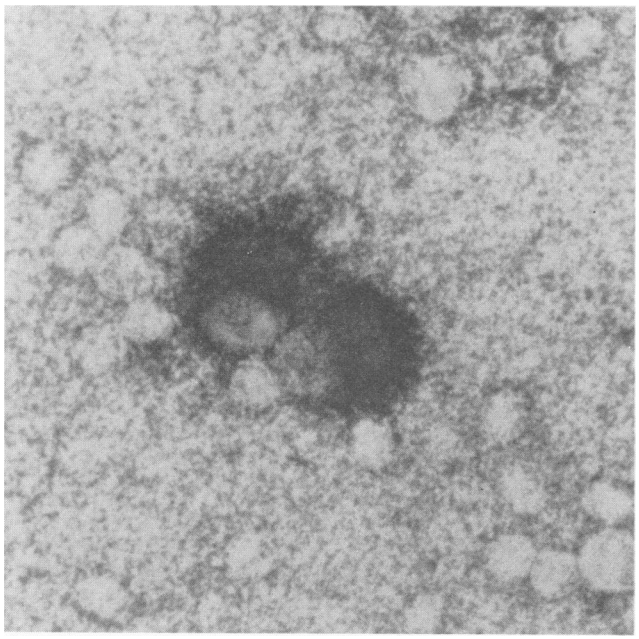

FIG. 3. Simultaneous detection of VZV RNA and neurofilament proteins in infected DRG cultures. The cells were fixed at 3 days postinfection, and double labeling was done with antineurofilament antibody revealed with a peroxidase-labeled second IgG. In situ hybridization was done on the same cells with *Bam*HI probes labeled with ^{35}S. Magnification, ×560.

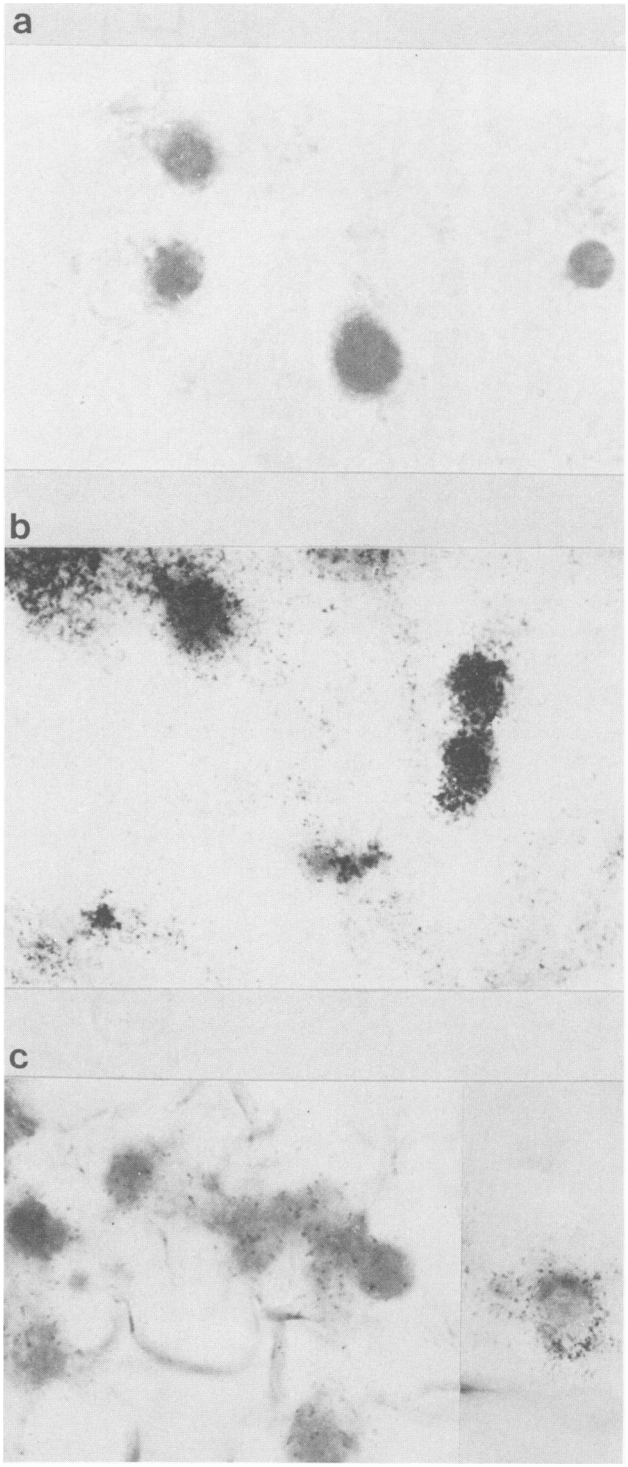

FIG. 4. Detection of VZV RNA in infected neurons by in situ hybridization with ^{35}S-labeled probes. (a) Control uninfected cells probed with a fragment that corresponded to IE gene 63. Infected cells (3 days postinfection) were probed with a fragment corresponding to IE gene 63 (b) and IE gene 62 (c). Original magnification, ×470. For the detection of ^{35}S-labeled probes, cells were dehydrated in graded ethanol, dipped in Ilford K2 autoradiographic emulsion, exposed for 2 to 5 days at 4°C, and developed in Kodak D19.

with those obtained when the infection was propagated by cocultivation with MRC5 cells. In addition, a similar transcription pattern was recorded by using cell-free virus infection and cocultivation, confirming that these genes are expressed in the neuronal cells.

Several relevant conclusions can be drawn from the data presented. A specific VZV infection of adult rat DRG neurons has been obtained by cocultivation with infected MRC5 cells or by cell-free virus. As shown by immunofluorescence, in situ hybridization, and simultaneous detection of VZV and neuronal protein, neurons are the only cell type which is infected in vitro, but VZV-specific antigens are not expressed in neurons. Viral nucleic acids are detected in infected neurons, and the hybridization signals increase with the time postinfection. At 6 days, almost half of the neurons are infected by VZV. In addition, no infectious virus and no antigens are released from the infected neurons, nor can they be recovered in the culture medium. These findings indicate that neuron-specific infection of adult rat DRG is persistent because (i) the viral genome is present, (ii) the complete infectious virus is not produced, and (iii) the number of neurons is maintained stable up to 10 days postinfection, showing that the infection process is not lytic. It remains now to determine the nature of the chemical, physical, or biological treatments which may be used to trigger virus replication.

The percentage of infected neurons visualized by in situ hybridization remained the same whatever the time of DRG culture before infection with VZV. The same efficiency of neuron infection at any time of culture suggests that infection is not mediated by a molecular event related to acute neuronal damage or regeneration. It would be interesting to know whether neuronal infection is random or whether there is selectivity in terms of the neuronal subpopulation (8), considering for instance the neurotransmitter phenotype (J. Schoenen, P. Delrée, P. Leprince, and G. Moonen, J. Neurosci. Res., in press).

The analysis of VZV transcripts by in situ hybridization has shown the presence of RNA transcripts from regions corresponding to the three temporal gene classes (IE, early, and late), suggesting that transcription is not blocked in the IE or early stage of infection. Moreover, hybridization signals were stronger when neurons were probed with a fragment corresponding to IE gene 63, indicating an active transcription of this gene in infected neurons. Further experiments are required to fully characterize the complete VZV genomic transcription in infected rat DRG cultures either by in situ hybridization or by Northern (RNA) hybridization. Like VZV, HSV-1 establishes latent infection in neurons of sensory ganglia and expresses IE genes which encode infected-cell polypeptides (ICP) (17, 19). HSV ICP4 protein has been detected in latently infected ganglia and shown to be a regulatory protein in vivo and in vitro (1). ICP0 RNA has also been detected in neuronal nuclei of latently infected murine (19) and human ganglia from cadavers (5). These transcripts overlapped and had a polarity opposite to that of mRNA of the ICP0 gene and could be referred to as an antisense transcript. The authors suggest that these transcripts may play a role in maintaining the latency of HSV. The situation is quite different for VZV, because no homolog of this HSV-1 ICP0 gene has yet been described in its genome. DNA sequence analysis (7) and recent reports (11) have identified VZV genes similar to HSV IE genes. VZV gene 62 encodes a protein of 175 kilodaltons which is the functional analog of HSV-1 ICP4. VZV gene 63 displays a sequence homology to HSV-1 ICP22 (30 kilodaltons). VZV proteins of similar size have been detected in explanted human ganglia (21) and may be related to VZV genes 62 and 63. Moreover, in situ hybridization revealed the presence of gene 63 RNA in the same ganglia. These results suggest that IE genes 62 and 63 expressed during latency may play a role in establishing and maintaining VZV latency in humans.

In a recent report (6), Croen et al. studied the cellular localization and viral transcription pattern of acute and latent VZV infection of human sensory nerve ganglia by in situ hybridization. The authors claimed that latent VZV infection involves nonneuronal cells and that multiple but not all VZV genes are transcribed. Transcripts have been detected with unidirectional probes from *Bam*HI-E (ORF4), *Bam*HI-J (ORF63), and *Eco*RI-B (ORF29, ORF30, and ORF31). During varicella disease, both neuronal and nonneuronal cells are infected and all regions of the VZV genome appear to be expressed.

In VZV infection of rat sensory neurons, we have detected transcripts of IE, early, and late genes but more abundant transcripts of IE gene 63, suggesting that the latter may play a role in repressing a productive infection in rat sensory neurons.

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