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Latency *In Vitro* of Varicella-Zoster Virus in Cells Derived from Human Fetal Dorsal Root Ganglia

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ABSTRACT. A potential *in vitro* model of varicella-zoster virus (VZV) latency was developed. Dissociated human dorsal root ganglion cultures were infected with VZV and maintained for 1 wk in the presence of bromovinyl arabinosyl uracil, a potent inhibitor of VZV. Seven to 21 d after removing the inhibitor (≥14 d after infection), the cells were trypsinized, passed to monolayers of human embryonic lung fibroblasts, and observed for VZV reactivation as indicated by typical cytopathic effects and the appearance of VZV antigens. VZV reactivated from 56% of the cultures containing both neurons and satellite cells but not from cultures specifically enriched for either neurons, satellite cells, or ganglion-derived fibroblasts. The failure to isolate VZV from cell suspensions that were sonicated before cocultivation with fibroblasts indicated that infectious VZV was not present before reactivation. Moreover, immunohistochemical and immunoprecipitation studies revealed no VZV-specific antigens in any cultures before the reactivation stimulus. VZV antigens were detected after trypsinization and cocultivation. These findings suggest that cultures containing both neurons and satellite cells provide a model system for VZV persistence that possesses many properties of a latent infection. (*Pediatr Res* 32: 699–703, 1992)

Abbreviations

VZV, varicella-zoster virus
HSV, herpes simplex virus
NGF, nerve growth factor
HELF, human embryonic lung fibroblast
CPE, cytopathic effect
DRG, dorsal root ganglion
DMEM, Dulbecco's modified essential medium
EMEM, Eagle's minimal essential medium
FUDR, fluorodeoxyuridine
BVaraU, bromovinyl arabinosyl uracil
pfu, plaque forming units

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VZV, which produces latent infection of human DRG during primary infection (varicella), can reactivate many years later to produce herpes zoster. The sites of latency were initially assumed to be the DRG and trigeminal ganglia, on the basis of the characteristic dermatomal distribution of herpes zoster (1–3) and the detection of VZV within the ganglia during the acute phase of herpes zoster (4, 5). However, numerous attempts to recover VZV from DRG and trigeminal ganglia of adult cadavers have all failed, in contrast to the frequent recovery of HSV from trigeminal and sacral ganglia (6, 7).

These sites of VZV latency were established, finally, by detecting VZV nucleic acids in human ganglia harvested at autopsy from individuals who died without active infection (8–11). However, *in situ* hybridization studies using VZV-specific probes have yielded conflicting data concerning the cellular location of the latent VZV and the prevalence of infected cells within these ganglia (8, 10, 12, 13).

A model of VZV latency was approached in adult rats by s.c. paraspinous injection of virus and its subsequent detection in neurons by *in situ* hybridization. VZV was also reported to be reactivated from ganglion cells from these rats upon trypsinization and cocultivation with permissive fibroblasts (14). These findings seem surprising because the rat is not readily infected by VZV. Moreover, their relevance to latency in man is uncertain.

We have investigated an *in vitro* model of VZV latency, based on an *in vitro* model of HSV latency, which appears to mimic many of the features of HSV latency noted in humans and infected animals (15). In this HSV model, dissociated DRG neuronal cultures prepared from rat or human fetuses are infected with HSV in the presence of NGF, and acyclovir is included to limit productive infection. Acyclovir is removed 7 d after infection. HSV-latent infection, as indicated by the absence of infectious virus or viral antigens, is maintained as long as cultures are maintained in the presence of NGF. Removal of NGF from the medium results in prompt and consistent reactivation. Based on some of the principles of the HSV model, we have proposed and characterized an *in vitro* model of latent VZV infection in cells derived from human fetal DRG.

MATERIALS AND METHODS

VZV inoculum. Cell-free VZV was prepared by infection of monolayer cultures of HELF, passage 10–18, maintained with DMEM supplemented with 10% FCS. After characteristic CPE

were observed, the cells were scraped, sonicated (10-mL aliquots; Braun-Sonic no. 1510; 100 W for 30 s; 4°C), and centrifuged (1500 rpm for 10 min), and the supernatant was stored at −70°C. These VZV stocks had a titer of 4×10^5 pfu/mL.

Neuron cultures. Sensory neuronal cultures were prepared from DRG of human fetuses that were at 8–15 wk of gestation. The tissue was obtained from already-scheduled therapeutic abortions. Written, informed consent was obtained for use of the fetal tissue for scientific purposes. The DRG was dissected from the spinal cord and enzymatically digested for 30 min at 37°C with 1 mg/mL of collagenase (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) in L-15 medium (Gibco Laboratories, Grand Island, NY). DRG were rinsed with L-15 medium containing 5% newborn calf serum, mechanically dissociated in EMEM, and plated onto 24-well multidishes coated with rat-tail collagen.

Neuronal cultures were maintained in EMEM containing 10% newborn calf serum and 50 ng/mL mouse 2.5s NGF. Neuronal cultures were treated with 20 μM FUDR (Sigma, St. Louis) for 7–10 d after plating to inhibit growth of nonneuronal cells (15, 16). FUDR was removed 12 h before virus inoculation. Wells typically contained 1×10^4 neurons. NGF was present in the medium unless otherwise indicated.

Satellite cell cultures. DRG were harvested as described above, but the cells were maintained in DMEM plus 10% FCS in the absence of NGF and treated with cytosine arabinoside (10 μg/mL) for 48 of every 96 h to inhibit growth of fibroblasts (16). Seven to 10 d after plating, 35–50% of the cells stained with antibody against S-100 antigen. This is the percentage of nonneuronal cells that stain positively for S-100 antigen in sections of human ganglia harvested at autopsy (unpublished results). The S-100 antigen staining probably underestimates the actual number of satellite cells in the tissue culture because the expression of this antigen varies with the stages of differentiation (17, 18). The other cells in this culture have the morphologic appearance, using low-magnification microscopy, of fibroblasts; no neurons were evident.

Mixed (neurons and satellite cells) culture. DRG cells were treated with EMEM plus 10% FCS and NGF. FUDR was added for 48 of every 96 h and removed before virus inoculation.

DRG-derived fibroblasts. DRG cells were maintained with DMEM supplemented with 10% FCS. Confluent monolayers were trypsinized and passed three times. Fibroblasts were the only cell type apparent in final cultures.

VZV infection of neuronal cultures. Twelve h before infection, neural cell cultures were treated with BVaraU (Squibb Institute for Medical Research, Princeton, NJ) at a final concentration of 0.29 μM or 2.9 μM, which approximated 10^3–10^4 times the median infective dose for VZV in fibroblasts (19). Cells were infected with 0.5–1.0 pfu/cell of VZV in the presence of BVaraU. Seven d after infection, the antiviral agent was removed by washing the culture five times with 1.0 mL of medium. The medium was changed every 2–3 d.

VZV reactivation. Seven to 21 d after removal of BVaraU (≥14 d after infection), cells were trypsinized and added to monolayers of HELF. Cells were inspected daily for 7 d; if there was no CPE, they were trypsinized and blindly passed to fresh HELF monolayers for an additional 14 d. In some experiments, the cell suspensions were sonicated, as described above for the VZV inoculum, before addition to HELF. An alternate procedure for reactivation was the addition of HELF directly to the neuronal cell cultures. The HELF cultures were inspected for 21 d for CPE and studied by immunohistochemistry or immunoprecipitation for the presence of VZV antigen.

Staining for VZV immediate-early and late proteins. Cell monolayers grown on glass coverslips were fixed with methanol/acetone (1:1) for 10 min at −20°C, air dried, and stored at −20°C. For antigen staining, the coverslips were treated for 20 min at room temperature with 1.5% normal horse serum in PBS, washed with PBS, and reacted with a monoclonal murine anti-VZV glycoprotein I (gp-I) antibody designated 4E6 (20), or a murine anti-VZV immediate-early protein (gene 62 product) antibody designated 2XF1 (21), for 30 min at room temperature. After washing the cultures with PBS, biotinylated horse anti-mouse IgG (0.5% in PBS; Vector Laboratories, Burlingame, CA) was added for 30 min at room temperature and then washed with PBS. Avidin-biotin horseradish peroxidase complex was added for 30 min and washed with PBS, and the bound peroxidase activity was detected with diaminobenzidine in the presence of H_2O_2 and nickel chloride (Vector DAB Substrate Kit; Vector Laboratories). The cells were counterstained with hematoxylin.

Radioactive Labeling of Neuronal Proteins and Immunoprecipitation. Cultures were placed in methionine-free minimal essential medium for 60 min and then labeled with [^{35}S]methionine (300 μCi/mL; sp act > 1000 Ci/mol; Amersham Corp., Arlington Heights, IL) for 3 h at 37°C. They were washed three times with PBS and disrupted in 4 mL of lysis buffer (0.02 M sodium phosphate, pH 7.6, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS). The lysates were kept on ice for 2 h and centrifuged at 40 000 rpm in a Beckman SW60 rotor for 2 h at 5°C. Supernatants were stored at −70°C. Cell lysates (0.5–0.7 mL) were subsequently incubated for 2 h at 4°C with 30 μL of a 10% formalin-fixed suspension of protein A-containing *Staphylococcus aureus* Cowan I (22). After centrifugation in a microfuge at 4°C for 5 min, VZV-specific proteins were immunoprecipitated at 4°C for 16 h by incubation with MAb (50–100 μL) prepared against VZV-encoded glycoproteins: gp I, gp II, and gp IV (23). Finally, 30 μL of a 10% formalin-fixed *S. aureus* was added, and after 2 h at 4°C, absorbed immune complexes were washed three times with lysis buffer and suspended in 20 μL of TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA). After addition of 10 μL of 3× sample buffer (150 mM Tris-HCl, pH 7.0, 6% SDS, 15% 2-mercaptoethanol, 0.01% bromophenol blue), the suspension was heated in boiling water for 4 min, cooled on ice, and analyzed by 10% SDS-PAGE as described previously (24).

Electrophoresis was carried out for 16 h at 50 V per gel at room temperature. The gels were stained with 50% methanol/7% acetic acid/2% Coomassie brilliant blue and destained in 10% methanol/7% acetic acid. Gels were processed for fluorography (En^{3}Hance; New England Nuclear Corp., Boston, MA), dried, and exposed to Kodak X-Omat AR film at −70°C.

RESULTS

VZV infection of cells derived from DRG. VZV produced an easily recognized, progressive lytic infection in neurons, satellite cells, mixed cell cultures and DRG-derived fibroblasts within 24 to 96 h (Fig. 1), as demonstrated previously (16). The CPE was similar to that seen with VZV infection of other human cell types, and the infected cells stained positively for VZV immediate-early (Fig. 2*B*) and late antigens (data not shown). Lysates from each of the acutely infected neural cell types contained VZV proteins detectable by immunoprecipitation (neurons shown, Fig. 3*B*; other types not shown).

VZV infection of DRG-derived cells in the presence of BVaraU. As expected, BVaraU (2.9 μM) completely prevented lytic infection. After removal of BVaraU and NGF, however, no CPE was observed, and no VZV antigens were detected during an observation period of 7 to 21 d (Figs. 2*C* and 3*C*). In control experiments, to exclude persistence of BVaraU in cells, uninfected cells were treated with BVaraU and washed as described for the model; these cells were capable of supporting VZV replication to the same extent as untreated monolayers. Furthermore, the medium left after the extensive washing procedure contained no residual inhibitory effect on VZV as assayed in HELF or in neural cells. BVaraU at a 10-fold lower concentration (0.29 μM) similarly prevented lytic infection without evidence of VZV replication after its removal.

Reactivation of VZV. Attempts to recover virus by cocultiva-

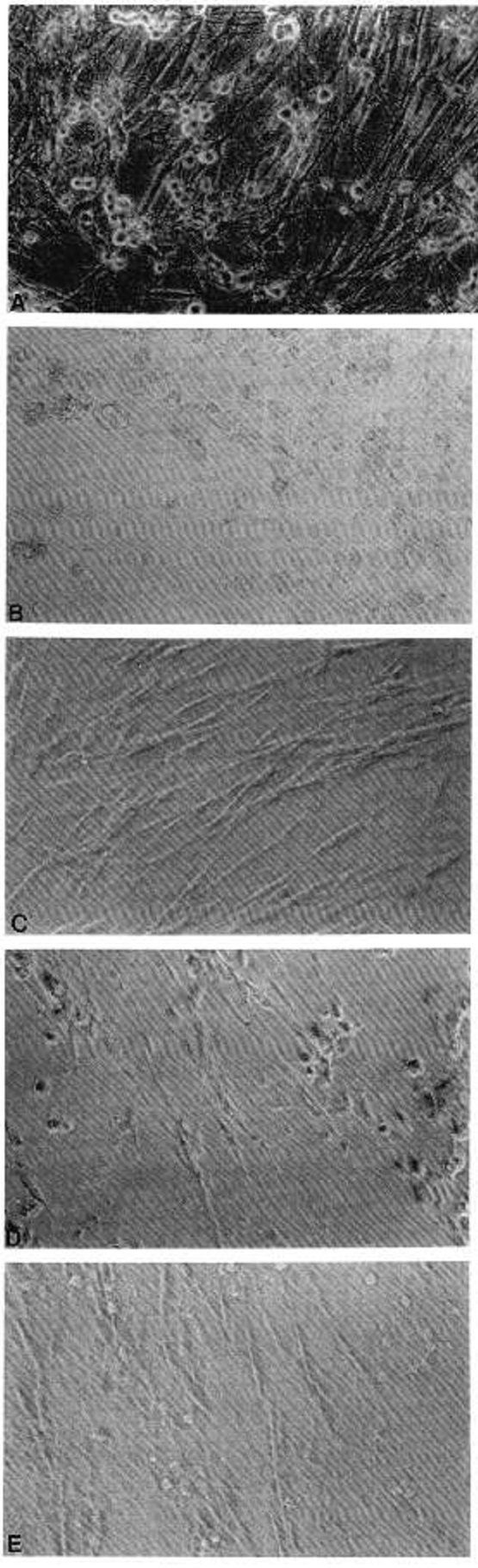

Fig. 1. VZV infection of DRG-derived cells (pictures taken at magnification power of 200×). *A*, Uninfected neurons; *B*, neurons infected with VZV (72-h postinfection); *C*, uninfected satellite cells; *D*, satellite cells infected with VZV (72-h postinfection); and *E*, uninfected mixed (neurons and satellite cells) culture.

tion were made from each of the different types of DRG-derived cell cultures that were infected with VZV in the presence of BVaraU and then tested 7–21 d after removal of BVaraU. The only type of cell culture from which VZV was recovered was that containing both neurons and satellite cells. The virus was recovered in 26 of 46 attempts (56%) after trypsinization and cocultivation with HELF cells (Table 1). Less frequently, in three of 20 attempts (15%), VZV was recovered after addition of HELF cells directly to candidate cultures. The presence of the virus was evident by typical CPE and confirmed by detection of VZV antigen (Figs. 2*D* and 3*D*; *additional bands* seen in Fig. 3*D* are occasionally seen in cells infected with VZV and harvested at various time periods after infection). Before cocultivation, the cultures did not contain infectious virions, because sonication of the cells yielded no plaques when the lysates were added to permissive cell monolayers (Table 1). The rate of VZV recovery after sonication under conditions used in these experiments was one cell-free infectious virion for every twelve infectious centers present in the culture before sonication. VZV reactivation was not influenced by the presence or absence of NGF after removal of BVaraU (data not shown).

DISCUSSION

The mechanism of VZV latency and reactivation is unclear. A major obstacle to an understanding of VZV latency is the lack of reproducible *in vitro* or *in vivo* models that mimic natural infection. VZV is fastidious and difficult to work with in the laboratory; its ability to infect nonhuman tissues and small mammals is very limited.

Our attempt to establish an *in vitro* model of VZV latency is based on a similar HSV model (15). Our experiments, however, differed in several respects from those of Wilcox *et al.* First, BVaraU was used to inhibit VZV replication, inasmuch as BVaraU is a far more potent inhibitor of VZV than is acyclovir (19). Secondly, nonneuronal cells were included in the experiment because of evidence indicating that such cells may be sites of latency for VZV (8) and HSV (25). Finally, reactivation was not influenced by the presence or absence of NGF.

Each of the neural cell types studied was readily infected with VZV (Fig. 1) (16), but VZV replication was completely inhibited by BVaraU. Removal of BVaraU from infected cultures after 7 d did not result in spontaneous VZV reactivation. Typical CPE and VZV-associated antigen expression were not detected during 7–21 d of observation after removal of BVaraU. The lack of VZV expression during this observation period was not due to persistence of BVaraU in the medium for several reasons: *1*) the median infective dose of BVaraU for VZV, as tested in our laboratory, is at least 100-fold higher in neurons than in HELF (unpublished data); *2*) when we used a lower concentration of BVaraU (0.29 μM), the same results were obtained; *3*) cultures were washed exhaustively and refed periodically; *4*) the medium left after the washing procedure did not inhibit VZV replication in HELF or in neural cells; *5*) BVaraU-treated cells, which were washed and maintained as described for the model, were capable of being infected with VZV; and *6*) individual cell types did not yield VZV, whereas mixed cultures maintained under the same conditions did yield VZV.

The cultures did not contain infectious virus, as demonstrated by the failure to recover VZV after sonication. In our reconstruction experiments, the recovery of VZV with the sonication conditions used was 8.5%. Therefore, the probability, by chance alone, of not recovering complete virions present in these cultures at the time of sonication is 11/12 for each virion. However, because we routinely recovered more than 10 pfu/well after successful reactivation attempts with mixed neuronal and satel-

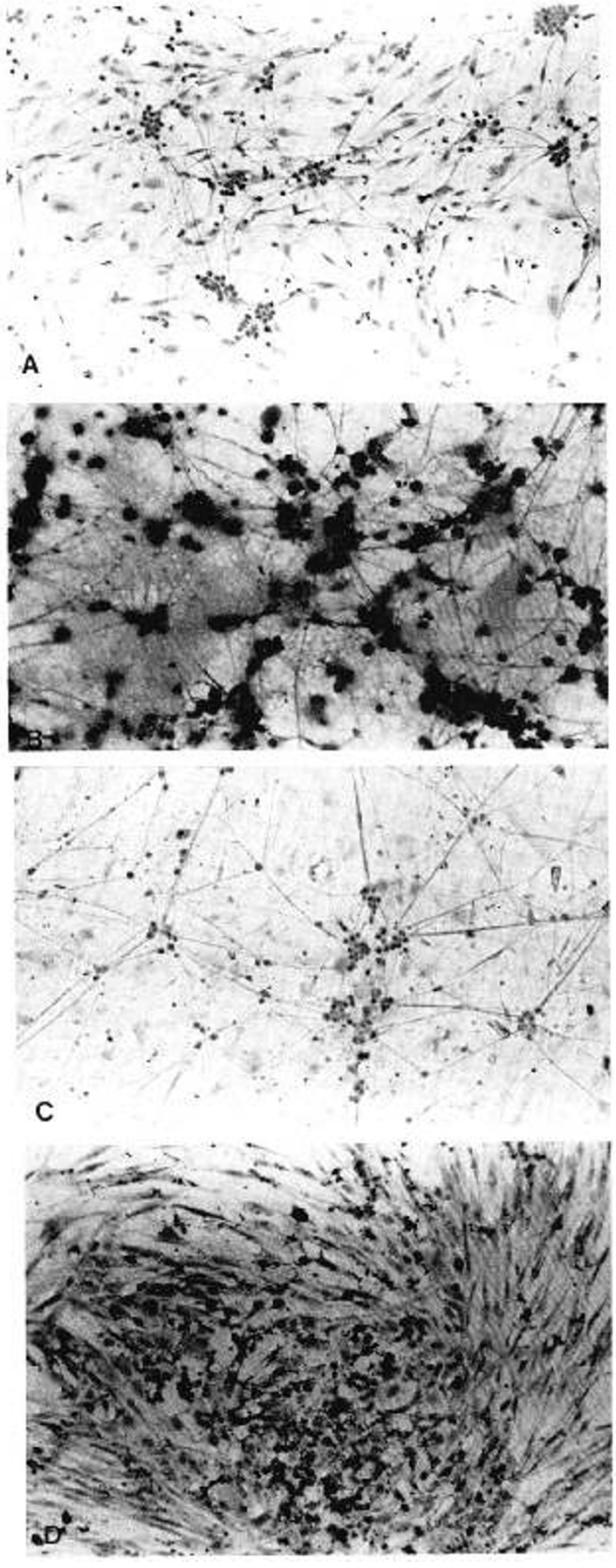

Fig. 2. Immunohistochemical detection of VZV immediate-early antigen in DRG-derived cells infected with VZV (pictures taken at magnification power of 80×). *A*, Mixed culture of neurons and satellite cells before infection with VZV; *B*, mixed culture of neurons and satellite cells infected with VZV (48-h postinfection); *C*, mixed culture of neurons and satellite cells latently infected with VZV (BVaraU removed for 8 d); and *D*, VZV reactivating in mixed culture after trypsinization and cocultivated with HELF; cell fixed 5 d after cocultivation.

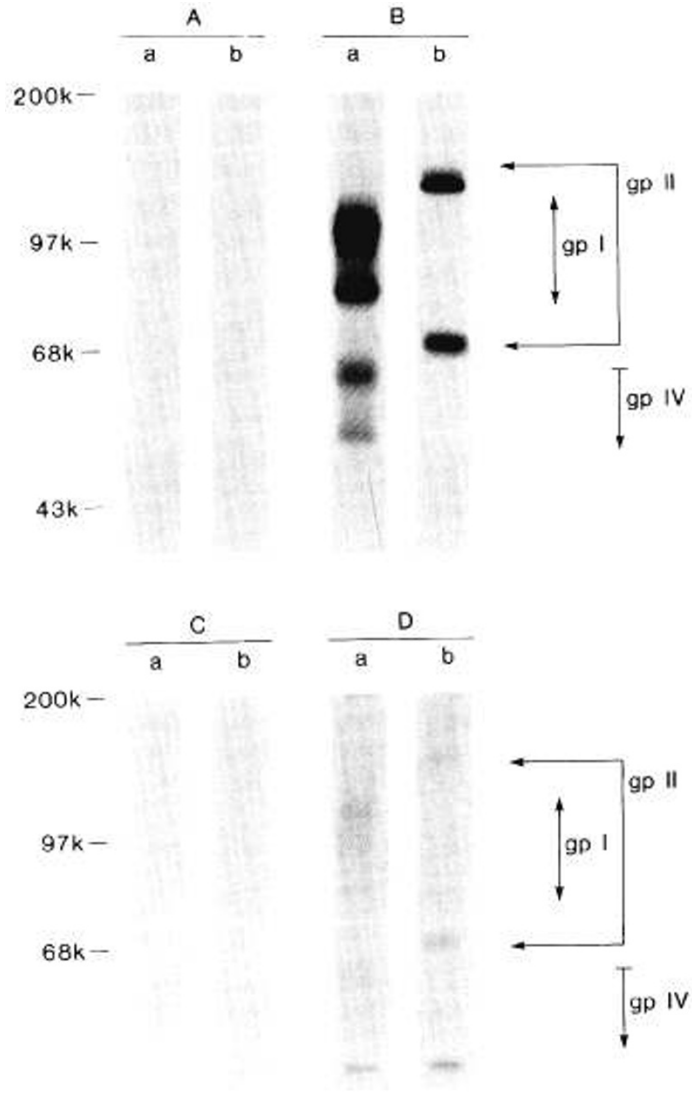

Fig. 3. Detection by immunoprecipitation of VZV antigens in DRG-derived cells infected with VZV. Neural cell lysates were immunoprecipitated with MAb against VZV gp I and gp IV (*lanes a*) and gp II (*lanes b*). *A*, Uninfected neuronal cells; *B*, VZV-infected neuronal cells (cells harvested 48 h postinfection); *C*, mixed culture latently infected with VZV (BVaraU removed for 10 d); and *D*, mixed culture after VZV reactivation (cells harvested 5 d after cocultivation with HELF).

lite cells and attempted recovery after sonication from 10 separate wells, the probability of losing all the virions by sonication alone in 10 different wells is $(11^{10}/12^{10})^{10} = 0.00017$.

When mixed cultures containing both neurons and satellite cells were cocultivated with permissive HELF cells, VZV was recovered (Table 1). This occurred most frequently (56%) when the cultures were trypsinized before passage to permissive HELF monolayers. VZV was not recovered when the initial infection was undertaken in fibroblasts, neurons, or satellite cells alone. Successful reactivation from mixed cultures could be achieved as long as 21 d after removal of BVaraU.

A unique feature of these experiments was the reactivation of VZV only from mixed cultures of neurons and satellite cells but not from the individual cell cultures studied. Satellite cells provide mechanical support for neurons and influence the metabolism and microenvironment of ganglionic neurons (26). These cells proliferate *in vitro* in response to neuronal damage (27). In addition, they enhance neuronal survival, growth, and neurotransmitter content (28). We speculate that an interaction between neurons and these nonneuronal cells is required to establish and/or maintain the persistence of VZV in our model, and

Table 1. *Reactivation of VZV from DRG-derived cells after infection in presence of BVaraU*

Type of DRG-derived cell culture*	Wells infected with VZV	Wells with VZV reactivation
Fibroblast	14	0
Neurons	35	0
Satellite cells	19	0
Neurons + satellite cells		
Trypsinized/passed to HELF	46	26†
HELF added directly to well	20	3
Sonicated/passed to HELF	10	0

* Cultures containing almost pure populations of the cell type or mixed cell type listed were infected with VZV as described in Materials and Methods. Fibroblasts, neurons, or satellite cells were passed to HELF monolayers for detection of VZV. Mixed cells (neuron plus satellite cells) were evaluated for the presence of VZV as indicated.

† The percentage of positive wells differs from all other experimental conditions with a p value <0.005 (χ^2).

that trypsinization of the mixed cultures disrupts this intercellular relationship, resulting in reactivation of VZV.

This concept is supported by the findings of Sadzot-Delavaux *et al.* (14). In their *in vivo* rat model of VZV latency, VZV could be recovered from DRG only after trypsinization and not simply after cocultivation of the DRG in permissive cells. There are also clinical observations that may reflect an enhanced proclivity of VZV to reactivate after disruption of neuron-satellite cell interactions. For example, herpes zoster occurs with an increased incidence within X-irradiated dermatomes or within or contiguous to dermatomes containing certain tumors (29–31).

The present model appears to represent the establishment and, at least briefly, maintenance of a unique VZV infection. Based on current information, this model functionally mimics the features of latent VZV infection in humans; namely, the absence of viral replication or infectious virions in the tissue with putative latent infection, the absence of gene products in these tissues, and the potential for reactivation of the latent VZV. Further studies are necessary to characterize the pattern of genome expression and to determine whether the viral transcript pattern in the *in vitro* model resembles that which is emerging from ongoing studies of naturally infected human ganglia (8).

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