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Phosphonoacetic Acid Induced Persistence of Varicella-zoster Virus in Cell Culture[1] (40717)

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Disodium phosphonoacetate (PAA) inhibits the replication of varicella-zoster virus (VZV) (1), and of other herpesviruses (2–9). Although PAA exerts minimal effect on host cell DNA polymerases, it appears to interact with the viral-induced enzyme at the pyrophosphate binding site, allowing initiation of polynucleotide synthesis, but preventing addition of deoxynucleosides to the elongating nascent chain (4, 9–13). While the inhibition *in vitro* of herpes simplex virus (HSV) and cytomegalovirus (CMV) is reversible after 72 hr on removal of PAA (4, 5), VZV has not similarly been recovered (1).

Few attempts have been made to develop a model that permits persistence of nonreplicating VZV in cell cultures. Short-term persistence has been reported after use of interferon (14) or of iododeoxyuridine (15). In this communication we report the recovery of VZV after prolonged exposure of infected human foreskin cultures (HFS) to PAA. Further, in the absence of a productive infection, VZV specified antigen(s) were detected in PAA exposed cells 84–90 hr after inoculation.

Materials and methods. Virus, cells, reagents. Human foreskin (HFS) fibroblast cultures, prepared locally and maintained on Eagle's minimal essential medium (E-MEM) supplemented with 2% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin (P-S) were used for propagation of VZV; HFS cells of a single cell line that represented the 14th to the 30th passage were employed. The strain of VZV employed in these studies derived from an acute case of varicella and had been passaged approximately 50 times in human cells. Stock virus was prepared by inoculating HFS cultures (150-cm^2 flasks) with VZV-infected cells that had been frozen and thawed once. Seventy-two hours later, cultures were harvested with glass beads and the cells resuspended in a storage medium containing sucrose, KH_2PO_4, K_2HPO_4, Na-glutamate, and bovine albumin (SPGA) (16). Cell-free virus was obtained by sonicating suspensions of harvested cells. The procedure employed was determined by the final volume of cell suspension; for <1 ml, the microtip of Model W 200R Heat Systems sonicator (60% power, 45 sec) was employed, and for >1 ml, a Bronson 75-S sonicator (power setting 5, 4 amp, 10 sec) was used. The resultant suspensions were centrifuged at 1500 rpm for 15 min. For preparation of high-titer virus (10^6–10^7 plaque-forming units (PFU)/ml), sonicated supernatant was concentrated 10- or 20-fold with polyethylene glycol (PEG) 6000 (17, 18). Stock solutions of disodium phosphonoacetate (obtained from Abbott Laboratories, Chicago, Ill.) were prepared in E-MEM (10 mg/ml).

Virus plaque assay. Preliminary studies confirmed findings of others that a semisolid overlay was not necessary for assay by plaque counts (1, 16). HFS monolayer cultures (35-mm plates) were inoculated with 0.2 ml virus-infected cell suspensions or cell-free VZV, incubated for 1.5 hr at 37°C in a 5% CO_2 atmosphere, washed one time, and refed with 2.0 ml E-MEM containing 2% FCS, 0.225 *M* sodium bicarbonate, and 50–100 μg/ml gentamicin. Plaques were counted on the sixth or seventh day with an inverted microscope.

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Long-term cultivation of inoculated cultures. HFS cultures (25-cm^2 flasks) were inoculated with VZV-infected cells or with PEG-concentrated cell-free VZV at a multiplicity of infection (m.o.i.) of 0.1 PFU or less per cell; after adsorption for 2 hr, monolayers were washed and maintained with E-MEM containing 50 μg/ml PAA (E-MEM-PAA) with twice weekly changes of medium. At indicated intervals cultures were washed three times before adding medium without PAA, or were subcultured by adding 1.0 ml 0.25% trypsin in phosphate-buffered saline (PBS) for 10 min at 37°C. Trypsinized cells were centrifuged, resuspended in E-MEM-PAA containing 10% FCS, and divided between two 25-cm^2 flasks. PAA usually was removed from one of the infected subcultures when confluent monolayers formed (3–5 days), and the other subculture was continued with PAA. Control cultures were similarly handled in the absence of PAA.

Indirect immunofluorescent staining (IFA). HFS cultures (35-mm plates) were inoculated with 0.2 ml unconcentrated sonicated cell-free VZV at an m.o.i. of 0.02 or less per cell. After adsorption for 2 hr at 37°C, the inoculum was aspirated, cultures were washed and refed either with E-MEM to examine antigen expression during a productive infection or with E-MEM-PAA for study of early antigen(s) expressed during a nonproductive infection. Cultures, including uninfected control plates, were incubated for 72 hr and then harvested by incubation with 2.5% trypsin for 5 min at 37°C. Centrifuged cells were resuspended in 1.5 ml of the appropriate maintenance medium and 1 drop of the cell suspension was placed on each of eight areas (diameter, 6 mm) circumscribed on a glass slide (Toxoplasmosis slides, Bellco, Vineland, N.J.). The slide cultures were incubated overnight (12–18 hr) at 31°C in a humidified atmosphere containing 5% CO_2 (19). Slides were washed in 0.85% saline, air-dried, fixed in acetone for 15 min, and stored at −70°C. The IFA test has been described elsewhere (20). Two zoster convalescent sera, and zoster immune globulin (ZIG) were used as a source of antibody, while two varicella negative sera were used as controls. Rabbit antihuman immunoglobulin G was prepared and conjugated to fluorescein as described (21).

Results. Dose-related inhibition of VZV replication by PAA. The concentration of PAA which completely inhibited the induction of viral cytopathic effect (CPE) related to the m.o.i. per cell (Table I). Complete inhibition of VZV CPE was observed at a m.o.i. of 0.042 or less per cell in the presence of 25 μg/ml PAA. At an m.o.i. 10-fold higher and with 25–100 μg/ml PAA, discrete plaques were not observed, but specific degenerative changes were evident along the edges of the monolayer. This effect was not observed in uninfected PAA cultures with drug concentrations as high as 100 μg/ml. Concentrations of PAA as low as 10 μg/ml resulted in a 93% reduction in PFU when the input viral multiplicity was also low (m.o.i. = 4.2×10^{-4}). In experi-

TABLE I. INHIBITORY EFFECT OF PHOSPHONOACETIC ACID (PAA) ON VARICELLA ZOSTER VIRUS REPLICATION IN HUMAN FORESKIN CELLS[a]

Concentration PAA (μg/ml)	Multiplicity of infection (PFU/cell)[b]					
	4.2	0.42	0.042	0.0042	0.00042	0.000042
0	++	++	++	+	60 PFU	5 PFU
10	++	++	++	+	4 PFU	0 PFU
25	++	++	0	0	0	ND
50	++	++	0	0	0	ND
100	++	++	0	0	0	ND

[a] Duplicate HFS monolayer cultures (35-mm^2 plates) were inoculated with 0.2 ml of 10-fold dilutions of PEG-concentrated cell-free VZV; after adsorption for 1.5 hr, monolayers were washed, and then cultured for 6 days with appropriate concentrations of PAA, at which time plaques were counted.

[b] ++, Degenerative changes, no discrete plaques; +, discrete plaques, too many to count; PFU, average number plaque-forming units per culture; 0, no cytopathic effect; ND, not done.

ments described below, complete inhibition of viral CPE was consistently observed using 50 μg/ml PAA and an m.o.i. of 0.1 or less per cell. At this concentration of PAA uninfected cells could be subcultured and appeared to grow without overt evidence of toxicity.

Recovery of infectious VZV after long-term cultivation with PAA. Infectious virus was never recovered from HFS cultures infected at an m.o.i. of 0.1 or less per cell while cultivated in the presence of 50 μg/ml PAA. When inoculated cultures that had been maintained for long periods of time with medium containing PAA were shifted to drug-free medium, infectious virus was recovered. Further, when PAA-maintained inoculated cultures were subcultured in the continued presence of PAA and then shifted to a drug-free medium, infectious virus was also recovered. These observations are summarized in Fig. 1. In one case, the viral genome persisted in the absence of overt CPE for 163 days; this included maintenance through one subculture in the presence of PAA for a period of 95 days, followed by 68 days cultivation in drug-free medium before viral CPE appeared. The use of cell-free or of cell-associated virus as inoculum did not influence viral recovery.

The interval necessary for recovery of VZV in drug-free medium lengthened with prolonged exposure to PAA as indicated in Fig. 1. In cultures maintained with PAA less than 2 weeks, viral CPE appeared within 1 week after drug removal, usually in 4 to 5 days. In cultures maintained with PAA up to 7 weeks, viral CPE appeared within 15 days after removal of the drug, most often between 9 and 14 days. When cultures were maintained with PAA for 8 or more weeks, the latent period after drug removal varied considerably. In one instance it was as short as 7 days, and at the other extreme, it was as long as 14 weeks; however, in most cases (four of six cultures) viral CPE did not appear until 6 weeks after drug removal. Whether or not subcultures were made in the course of an experiment did not appear to influence the period between drug removal and appearance of virus.

In order to compare the biological properties of persistent VZV with the parent strain, the following studies were done. One VZV plaque that developed at 119 days was harvested. The virus had persisted through two subcultures with PAA for 70 days, and then in drug-free medium for 49 days. Cells from the plaque were used to inoculate four HFS cultures (35-mm plates); two were maintained at 37°C and two at 31°C. In order to obtain cell-free virus in quantities adequate for study, infected cells maintained at the two temperatures were each passed five times. The two VZV isolates thus derived from the plaque were compared with the parent strain in terms of plaque morphology at 31 and 37°C. Varying concentrations of each isolate were examined with respect to the effective concentration of PAA required for inhibition, as had been done with the parent strain. No differences in these biological

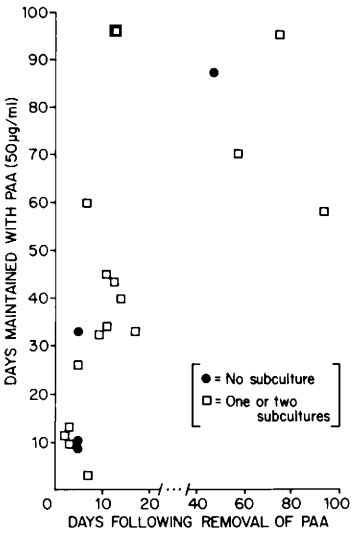

FIG. 1. Relationship between duration of exposure of cultured VZV to PAA and subsequent appearance of cytopathic changes following removal of the compound from the culture system. HFS cultures (25-cm² flasks) were inoculated with concentrated cell-free VZV at an m.o.i. of 0.1 or with VZV-infected HFS cells at a ratio to uninfected cells that ranged from 1:12.5 to 1:40, as established by plaque titration. Cultures were maintained with PAA and subcultured as described. Each symbol represents time of appearance of CPE in one culture related to duration of exposure to PAA and the interval following removal of the compound.

properties were noted between the isolates and the parent strain.

Quantitation of viral recovery. The amount of virus recovered from cultures after removal of PAA was inversely proportional to the duration of suppressive cultivation. Cultures (25-cm^2 flasks) were inoculated with PEG-concentrated cell-free VZV at an m.o.i. of 0.1 and maintained with 50 μg/ml PAA for various periods with or without subculture; at various intervals PAA was removed, and the number of PFU appearing per culture determined. After 10 days with PAA, only 1.4% of the original inoculum (PFU) was demonstrable. Continued cultivation in PAA for 22 to 25 more days resulted in a further loss of recoverable virus, with only 2 to 3 PFU appearing per culture. After 40 or more days cultivation with PAA, no more than one plaque per culture was recovered. No VZV was recovered from inoculated cultures maintained with PAA for more than 100 days.

Detection of VZV-specific antigen(s) in PAA-maintained cultures. When HFS cell cultures were prepared in PAA-free medium and examined 84 to 90 hr after inoculation with cell-free VZV, specific nuclear and cytoplasmic immunofluorescence was observed, as shown in Fig. 2a. VZV-Specified antigen(s) were also detected in similarly inoculated HFS cells that had been maintained in the presence of 50 μg/ml PAA, but the pattern of immunofluorescence was in marked contrast to that observed with productively infected cells. As illustrated in Fig. 2b, specific immunofluorescence was confined to an area immediately adjacent to the nuclear membrane. Rarely, specific immunofluorescence was also localized within the nucleus as well as the paranuclear area, as illustrated in Fig. 2c. Localization of immunofluorescence to the paranuclear area was observed repeatedly using two herpes zoster convalescent sera as well as ZIG, and was never seen in PAA-maintained infected cells reacted with VZV-negative sera. Fewer

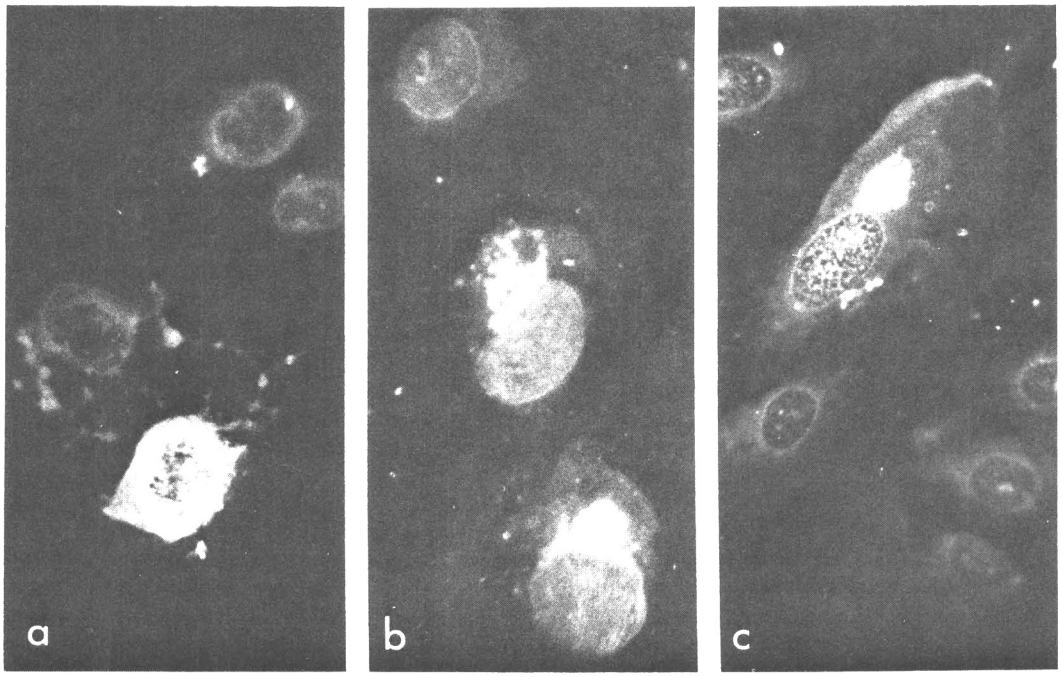

FIG. 2. Indirect immunofluorescent staining with herpes zoster convalescent serum of cultured HFS cells 84–90 hr after inoculation with cell-free VZV: (a) productive infection in cultures without PAA; (b) and (c) nonproductive infection in the presence of 50 μg/ml PAA.

cells expressing viral-specified antigen(s) were seen in PAA-maintained cultures than in matching drug-free cultures. Those VZV-infected cells exposed to PAA exhibiting specific fluorescence often appeared to be enlarged (Figs. 2b and c).

Discussion. These results document the prolonged persistence of VZV in cultured human cells maintained in medium containing PAA, and additionally demonstrate the appearance of viral-specified antigen(s) in such cells 84 to 90 hr after inoculation. With appropriate m.o.i. and in the presence of 50 μg/ml of PAA in the medium, VZV persisted in cell culture in a nonproductive state for many weeks. Viral CPE was never observed under these conditions. However, continued cultivation after omission of PAA from the medium resulted in VZV plaque production after varying periods of time. The period before virus became manifest after removal of PAA was in general proportional to the period of suppressive cultivation in the presence of PAA. The increased period of cultivation necessary for recovery of virus after prolonged exposure to PAA might reflect the presence of residual intracellular PAA with continued suppression of viral replication until degradation or discharge of the intracellular accumulation.

By immunofluorescent studies we detected antigen synthesis in PAA-maintained HFS cells 84–90 hr after inoculation with cell-free VZV. The observation that some of the cells exhibiting specific immunofluorescence also appeared to be enlarged suggests that such cells may be prone to autolysis which would account for the progressive decrease in recovery of virus on prolonged cultivation. After only 84–90 hr in PAA, fewer cells expressing viral antigen were detected in PAA-maintained cultures compared with cultures similarly inoculated and maintained in the absence of PAA. Therefore, the discrepancy between our findings and those of an earlier report (1), wherein VZV persistence and viral antigen in the presence of PAA could not be demonstrated after 72 hr, may be due to the use of a lower m.o.i. in the earlier study.

Early antigen synthesis in the absence of DNA synthesis has also been detected in PAA-exposed cells infected with Epstein–Barr virus (EBV) (6, 22), *Herpesvirus saimiri* (23), and cytomegalovirus (J. L. Waner, personal communication). In the present studies with VZV-infected cells maintained in PAA, specific immunofluorescence was generally localized in the cytoplasm, usually in the paranuclear area. Such viral-specified antigen may possibly represent induction of an Fc receptor (24). Since specific immunofluorescence was not observed in VZV-infected cells when reacted with VZV-negative sera, this possibility seems unlikely but cannot be ruled out.

Cytoplasmic localization of early antigen has also been observed in Raji cells abortively infected with EBV (25). The particular pattern of immunofluorescence so described correlated with the source of human sera in that the phenomenon could be shown with most sera from patients with Burkitt's lymphoma, but not with sera from individuals with infectious mononucleosis or nasopharyngeal carcinoma (25). It will be of interest to determine whether the particular pattern of immunofluorescence seen in PAA-maintained VZV-infected cells is restricted to only some, or all, convalescent varicella and zoster sera, and whether antisera experimentally produced in animals also have the capacity to reveal the paranuclear reactive material.

Summary. Varicella-zoster virus (VZV) persisted for weeks in a human cell culture system in the presence of phosphonoacetic acid (PAA) and could be recovered from the cultures after removal of the compound. The inhibition of VZV was dose dependant. VZV-Infected HFS cultures were maintained and subcultured in 50 μg/ml PAA with no evidence of cytopathic activity (CPE); when cultures were shifted to PAA-free medium, infectious virus was recovered. The period between removal of PAA and appearance of CPE was directly related to duration of exposure to PAA, and the amount of virus recovered was inversely proportional to the duration of suppressive cultivation. VZV-Specified antigen(s) were detected in the cytoplasm

(paranuclear region) of PAA-maintained HFS cells 84–90 hr after inoculation with cell-free VZV.

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