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Varicella-Zoster Virus: Isolation and Propagation in Human Melanoma Cells at 36 and 32°C

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Cell lines derived from human malignant melanoma tumors are susceptible to infection with varicella-zoster virus (VZV). Within 5 days after inoculation of vesicular fluid, cytopathic changes appeared in melanoma cell monolayer cultures that were incubated at either 36 or 32°C. The VZV isolates at the two temperatures were serially propagated by passage of trypsin-dispersed infected cells. A plaque assay was developed utilizing melanoma cell monolayers overlaid with nutrient medium containing carboxymethylcellulose. By this assay method, the growth cycle of a VZV isolate propagated at 36°C was studied and compared with that of another VZV isolate grown at 32°C. With equivalent infected-cell inocula at a ratio of one inoculum cell to eight uninfected cells, the yield of cell-free virus at an incubation temperature of 32°C was slightly higher than at 36°C, although the peak occurred 60 h, rather than 36 h, postinfection. It was also found that the titer of low-passage VZV propagated at 36°C was 0.5 to 1 log higher when assayed at 32°C rather than at 36°C.

In 1953, Weller reported the serial propagation of varicella-zoster virus (VZV) in roller tube cultures of human embryonic skin-muscle tissue and foreskin tissue (8). Weller and colleagues (9) further investigated the in vitro susceptibility of other human tissues to VZV infection. The diverse human cultures included myometrium, testis, postnatal kidney, amnion, and embryonic brain, all of which developed cytopathic effect (CPE) after inoculation with VZV. In their monograph on varicella virus (7), Taylor-Robinson and Caunt list more than 20 human and primate cell cultures (primary, diploid, and continuous) that have been reported as susceptible to VZV infection. Nevertheless, they conclude from their experience and that of other investigators that VZV can be readily propagated in but a few human tissues, mainly embryo fibroblast cells and amnion cells. In this paper, we describe the isolation and propagation of VZV in cell cultures derived from human malignant melanoma tumors. (The melanoma cell is the neoplastic counterpart of the melanocyte, which is derived embryologically from the neural crest). Special emphasis has been placed on a comparison of viral growth kinetics at incubation temperatures of 36 and 32°C. A preliminary report of the susceptibility of melanoma cells to VZV infection was previously presented (C. Grose and P. A. Brunell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, S386, p. 343).

MATERIALS AND METHODS

Tissue culture. Three pigment-producing cell cultures established from three different human malignant melanoma tumors were supplied by M. Bean (Sloan-Kettering Institute, New York, N.Y.). The derivation of these cell lines has been described previously (2). Chromosome analyses of several melanoma cell lines by Romsdahl and Cox revealed that the chromosome number may vary from hypodiploid to triploid (5). Melanoma cells were grown in Eagle minimum essential medium (MEM) supplemented with 2 mM glutamine, 1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 12.5% fetal calf serum. The cells were maintained in MEM with 2% fetal calf serum. Cultures were periodically checked for mycoplasma and were discarded if contaminated.

Source of virus. Vesicular fluid was obtained from three children: (i) an 8-year-old boy with herpes zoster (shingles), (ii) a 12-year-old girl with herpes zoster, and (iii) an 11-year-old boy with chickenpox. The two children with zoster demonstrated greater than 16-fold rises in VZV complement-fixing antibody; the child with chickenpox did not have serological studies.

All viral isolates were confirmed as varicella by checkerboard titration of dilutions of the isolates (antigen) against dilutions of VZV (Scott strain) antiserum and also herpes simplex (MacIntyre strain) antiserum prepared in guinea pigs (Microbiological Associates, Bethesda, Md.). In addition, melanoma cells infected by the viral isolates were examined for VZV-specific membrane antigen by the indirect immunofluorescent technique (10). Three antisera were used in

the latter test: (i) human VZV immune serum, (ii) human VZV nonimmune serum (titer <1:2 when reacted with human embryo lung fibroblasts infected with VZV-Ellen strain), and (iii) rabbit anti-herpes simplex (HFEM strain) antiserum.

Preparation of cell-free virus. Cell-free virus was prepared as described previously by Brunell (1). After the medium was aspirated from a 150-cm^2 tissue culture flask, fresh MEM (10 ml) was added, and the infected-cell monolayer was scraped from the surface with a rubber policeman. The cells were pelleted by centrifugation at 1,000 rpm for 10 min, suspended in 1 ml of sorbital buffer [10% sorbital in 0.05 M tris(hydroxymethyl)aminomethane (pH 7.4) and 0.001 M $MgCl_2$], and subjected to sonic treatment at 20 kHz for 30 s (Branson Sonifier model W 185D, equipped with a microtip). The disrupted cell suspension was centrifuged at 1,500 rpm for 15 min, after which the supernatant containing the cell-free virus was either titrated or stored at −70°C.

Virus plaque assay. Melanoma cell monolayers were trypsinized and resuspended in medium at a concentration of 2×10^5 cells per ml. One milliliter of cell suspension was placed into each well (16-mm diameter) of a 24-well plastic tray (Linbro Scientific, Hamden, Conn.). Serial dilutions of virus suspension (either infected cells or sonically treated preparations) were prepared in MEM, and 0.2 ml of each dilution was added to each of four to six wells containing nearly confluent monolayers from which the medium had been removed. After 1 h of adsorption at 36°C, the 0.2-ml inoculum was removed and replaced with 1 ml of MEM overlay maintenance medium (pH 7.6) containing 0.75% carboxymethylcellulose and 5% fetal calf serum. Cultures were incubated in a humidified 5% CO_2 incubator at 36°C. After 6 days, the wells were filled with an additional 1 ml of 10% Formol-saline. The fixed monolayers were stained with 0.1% crystal violet solution for 0.5 h, rinsed with tap water, and dried. After staining, the cells acquired a dark blue-purple color, whereas the viral plaques were reddish-purple and easily recognizable when counted with the aid of an inverted microscope (magnification, ×25). The virus titer is expressed as plaque-forming units (PFU) per milliliter of inoculum. The above plaque assay incorporates many features of the semi-micro-method described by Rager-Zisman and Merigan (4).

RESULTS

Isolation of VZV. Each of the three melanoma cell cultures was inoculated with vesicular fluid from one of the three children. At the same time, human embryo lung fibroblast cell cultures were similarly infected. Two sets of the infected cell cultures were incubated at 36°C, and the third set was incubated at 32°C. Within 3 to 5 days at both temperatures, the melanoma cells and human embryo lung fibroblast cells showed CPE. The CPE in the melanoma cells was characterized by small multinucleate cells at 36°C and by large syncytia at 32°C often containing more than 100 nuclei. After passage of trypsin-dispersed infected cells at both temperatures, the CPE remained syncytial in character. Two VZV isolates in melanoma cells were selected for further characterization. The virus strain from the 12-year-old with zoster was isolated at 36°C and designated 'VZV-36.' The virus strain from the child with chickenpox was isolated at 32°C and designated 'VZV-32.' Both strains were passaged in melanoma cells two to three times at their respective isolation temperatures in order to prepare infected-cell stock virus, which was stored at −70°C in MEM containing 10% fetal calf serum and 10% dimethyl sulfoxide. Although all three melanoma cell lines were susceptible to infection with VZV, for uniformity a single cell line, 'MeWo,' (2) was selected as a cell substrate (passages 20 to 35) for all further experiments.

Growth kinetics of varicella virus (VZV-36) in human melanoma cells at incubation temperature of 36°C. Melanoma cell monolayers were prepared in plastic disposable 150-cm^2 tissue culture flasks (Corning Glass, Corning, N.Y.). The monolayers were inoculated with melanoma cells infected with VZV-36 (passages 5 to 10), and the newly infected cells were harvested by trypsinization when the CPE covered 50% of the monolayer. These cells were used immediately as inocula to infect other melanoma cell monolayers at a ratio of one cell from an infected culture to eight uninfected cells. The infected-cell inoculum was suspended in 5 ml of MEM, sufficient to cover the bottom of the flask, and incubated for 1 h at 36°C, after which time the inoculum was removed and replaced with fresh MEM containing 12.5% fetal calf serum. At 12-h intervals after infection, the 25-ml culture medium was removed from one of the flasks and centrifuged for 1 h at 24,000 rpm in an SW27 rotor. After sedimentation, the supernatant was discarded, and the pellet was suspended in 1 ml of sorbitol buffer and subjected to sonic treatment for 30 s. The total amount of infectious cell-free virus in the medium overlying an infected monolayer was then determined by plaque assay (Fig. 1). The infected-cell monolayers were harvested by scraping the cells into 10 ml of MEM. After a wash, the infected cells were resuspended in 1 ml of sorbitol buffer and subjected to sonic treatment as described in Materials and Methods. All portions of cell-free virus, including the final time point, were stored at −70°C and then simultaneously assayed. To determine the titer of infected cells, a duplicate set of infected monolayer cultures were harvested by trypsinization at the same intervals. The cells were suspended in 1 ml of MEM and immediately plated on melanoma monolayers. Figure 1 correlates the extent of CPE with the titers of infected-cell and cell-

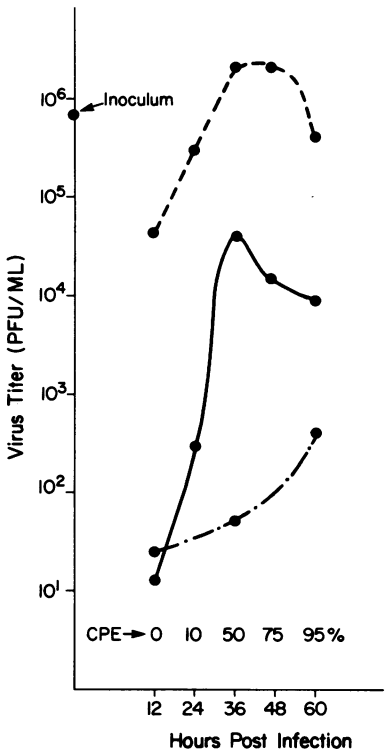

FIG. 1. *VZV infection at 36°C. Replicate melanoma cell monolayer cultures (150 cm²) were infected at a ratio of one inoculum cell to eight uninfected cells. The inoculum consisted of trypsin-dispersed cells from a VZV-infected monolayer with 50% CPE. Titers of infected cells (-----) and cell-free virus (——) were determined by plaque assays incubated at 36°C. Total infectious virus (· - · -) in the medium overlying the infected monolayers was assayed after ultracentrifugation of the medium (100,000 × g for 1 h), suspension of the pellet in 1 ml of sorbitol buffer, and sonic treatment. CPE, Cytopathic effect at each time point.*

free virus in the 150-cm² monolayers. Cell-free virus was first observed 12 h after infection and reached highest titer (>10^4 PFU/ml) 36 h postinfection, when the CPE involved 50% of the monolayer. Similarly, the titer of infected cells was highest between 50 to 75% CPE.

Assay of VZV-36 cell-free virus at 36 and 32°C. Human melanoma monolayers were infected with VZV-36 as described above and incubated at 36°C. At 12-h intervals after infection, the infected monolayers were harvested, and cell-free virus was prepared as described in Materials and Methods. At each time point, serial dilutions of the sonically treated virus preparations were inoculated into monolayers of two multiwell trays. One of the two trays was incubated at 36°C, as described for the growth curve at 36°C. The other tray was placed at 32°C for adsorption of the viral inoculum and also incubated at 32°C for the entire 6 days of incubation. The titer of VZV-36 cell-free virus at all time points was 0.5 to nearly 1 log higher when assayed at 32°C than at 36°C (Fig. 2).

Growth kinetics of varicella virus (VZV-32) in human melanoma cells at an incubation temperature of 32°C. A melanoma cell monolayer infected with VZV-32 (passages 5 to 10) and incubated at 32°C was trypsinized when CPE reached 50%. Melanoma cell monolayers (150 cm²) were inoculated with infected cells under the same conditions as described for VZV-36 infection, i.e., at a ratio of one inoculum cell to eight uninfected cells, but both the adsorption (1 h) and subsequent incubation were performed at 32°C. At 12-h intervals, the infected monolayers were harvested in the same manner as described for VZV-36 infections. During the first 36 h after infection, cell-free virus was not observed in the VZV-32-infected cell monolayers (Fig. 3A). At 48 h postinfection, when CPE was about 50%, the titer of cell-free virus was less than 100. Only after 72 h, when CPE covered more than 90% of the monolayer, was the highest yield of cell-free virus obtained (>10^4 PFU/ml). The number of infected cells was above 10^6 during most of the time course.

When the growth curves at the two temperatures (Fig. 1 and 3A) were compared, it was apparent that the ratio of PFU of cell-free virus to plaque-forming cells was much higher in the

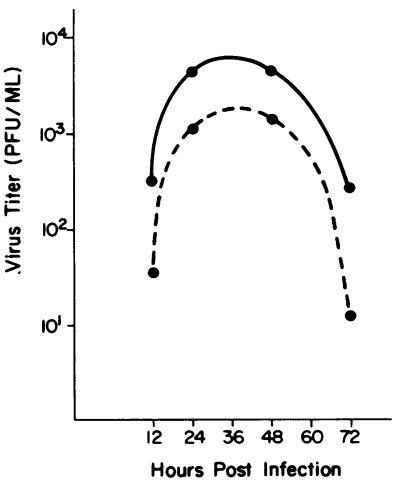

FIG. 2. *VZV infection at 36°C. Melanoma monolayers were infected as described in legend to Fig. 2. At each time point, cell-free virus was prepared and simultaneously titrated in two plaque assays, one of which was incubated at 36°C (-----), while the other was incubated at 32°C (——).*

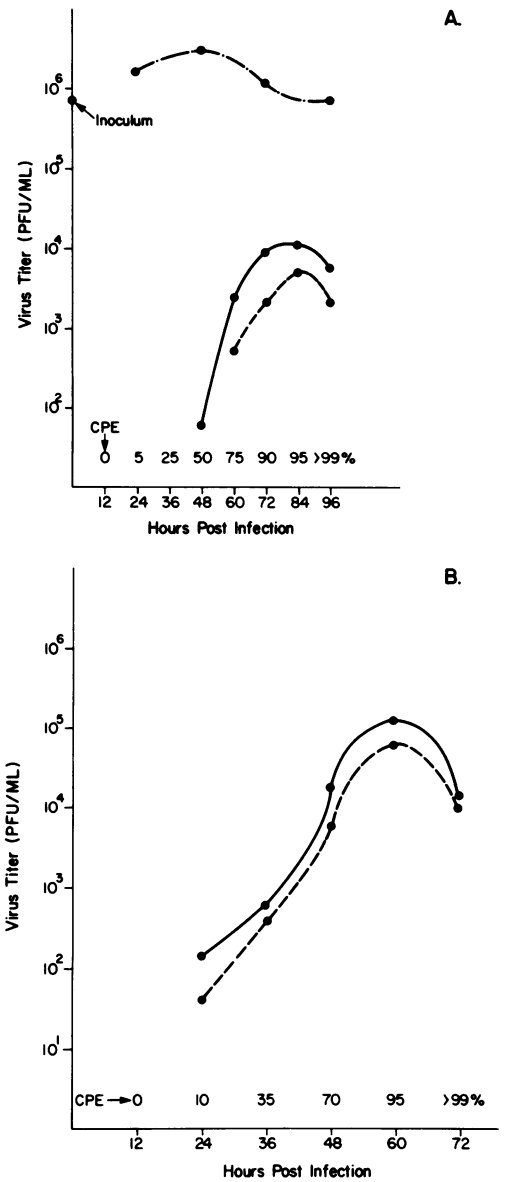

FIG. 3. *VZV infection at 32°C. Replicate melanoma cell monolayer cultures (150 cm²) were infected at a ratio of one inoculum cell to eight uninfected cells. The inoculum consisted of trypsin-dispersed cells from a VZV-infected monolayer with (A) 50% CPE or (B) 95% CPE. The titer of infected cells (· – · –) was determined by plaque assay at 32°C. The cell-free virus preparations were simultaneously assayed at incubation temperatures of 32°C (——) and 36°C (-----). CPE, Cytopathic effect at each time point.*

VZV-36-infected culture with 50% CPE than in the VZV-32-infected culture with similar CPE. The infected-cell inoculum in the 32°C culture, therefore, contained far less infectious virus per infected cell than did the inoculum at 36°C. Because of this discrepancy, a second set of growth curves were performed at 32°C with inocula consisting of infected cells from a trypsin-dispersed culture with advanced CPE. The latter inoculum, with more infectious virus per infected cell, elicited a higher yield of cell-free virus (>10^5 PFU/ml) at an earlier time point (60 h postinfection) (Fig. 3B). As seen in previous experiments, the titers in simultaneously prepared plaque assays incubated at 36°C were lower than those at 32°C (Fig. 3).

DISCUSSION

These studies demonstrate that human malignant melanoma cells are susceptible to infection by VZV. As with VZV infection in more conventional cell systems, such as human embryo lung fibroblast cells, the virus is predominantly cell associated, with little spontaneously appearing cell-free infectious virus in the culture medium. However, melanoma cells are an acceptable alternative to human embryo fibroblast cells as a cell substrate for laboratory studies of VZV. These cells are hardy, multiply rapidly, and have an unlimited life span (5). In the plaque assay, melanoma cells tolerate a carboxymethylcellulose overlay without developing nonspecific toxic changes. The assay can be read easily at 6 days after infection because the viral foci are so distinctive after staining with crystal violet.

A primary objective of this study was to determine when maximal yields of cell-free virus occurred after VZV infection of melanoma cell monolayers. To accomplish this goal, the growth kinetics of VZV infection were studied at an incubation temperature of 32°C as well as at 36°C. The conditions of infection as defined by Schmidt and Lennette for VZV infection of human fetal diploid lung cells (6) were also found to be optimal for VZV infection of melanoma cells incubated at 36°C. In both cell systems, the maximal yield of infectious cell-free virus occurred 36 h after inoculation with VZV-infected cells. However, an interesting phenomenon was observed when the sonically treated (cell-free) virus preparation derived from VZV propagated at 36°C (VZV-36) was simultaneously assayed at 36°C and 32°C. The titer of the same virus inoculum was higher by 0.5 to 1 log when the plaque assay was incubated at the lower temperature. This same observation was recently made by Hondo and colleagues (3), who found that a VZV inoculum formed about twice as many plaques in a virus assay (with human embryonic lung fibroblast monolayers) incubated at 31°C rather than at 37°C.

We also isolated and serially propagated VZV in melanoma cell cultures incubated only at

32°C. In contrast to the infections at 36°C, the highest yield of cell-free virus at 32°C was found in cell cultures with far advanced CPE. In addition, the titer of the sonically treated viral preparations at 32°C was higher than that from a comparable infection carried out at 36°C. These results suggest that VZV may be a naturally occurring temperature-sensitive virus whose optimal temperature for growth is below 36°C. If so, viral replication would improve with a shift in incubation temperature from the conventional 36 to 37°C down to 32°C. Such a temperature restriction would explain the consistently higher titers of both VZV isolates (VZV-36 and VZV-32) when assayed at 32°C than at 36°C. Temperature sensitivity is also an explanation for the presence of infectious virus in the superficial cutaneous vesicles which characterize chickenpox and zoster. Since serial passage of VZV at 36 to 37°C may select for temperature-resistant progeny, further studies with low-passage virus are required to define the temperature sensitivity of VZV.

ACKNOWLEDGMENTS

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