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Virus Replication and Localization of Varicella-Zoster Virus Antigens in Human Embryonic Fibroblast Cells Infected with Cell-Free Virus

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When human embryonic fibroblast cells were infected with cell-free varicella-zoster virus, virus replication began between 8 and 14 h postinfection, and 4 more h werp required for the virus to infect neighboring cells. Virus-specific antigens were traced by the anticomplement immunofluorescent antibody technique. Virus antigen was first detectable 2 h postinfection in the cytoplasma, and diffuse fluorescence was observed in the nucleus as early as 4 h after infection. The nuclear fluorescence got brighter and cytoplasmic fluorescence was observed at 14 h postinfection. The spread of virus to the neighboring cells was recognized in 18 h postinfection. In the period of 24 to 48 h, antigens were seen at the nuclear membrane region and in the cytoplasma. Very strong fluorescence was restricted mainly to the nucleus, when phosphonoacetic acid or cytosine arabinoside was added to the infected cultures and the cells were incubated for 48 h.

Since varicella-zoster virus (VZV) remains closely cell associated throughout the course of infection, it is difficult for investigators to obtain cell-free virus of high titer. This fact has hampered biological and immunological studies on this virus. The study of varicella virus antigens in tissue culture by immunofluorescent antibody was first reported by Weller and Coons (20). Several reports (5, 11, 15) appeared after this work, the investigators of which have attempted to use infected cells for immunofluorescent antibody study. However, it is necessary to use cell-free virus for studying the localization of VZV antigens and the time course of virus growth.

In this report, the localization of virus antigen was followed with the anticomplement immunofluorescence technique by using the method of cell-free virus infection. The effects of phosphonoacetic acid (PAA) and cytosine arabinoside (Ara-C) on the expression of VZV antigens in cells are also described in this report.

MATERIALS AND METHODS

Cell cultures. Human embryonic fibroblast (HuEF) cell cultures were grown in Roux bottles using Eagle minimum essential medium and medium 199 supplemented with 10 or 3% fetal calf serum for growth or maintenance media, respectively. All media used contained 100 U of penicillin and 100 μg of streptomycin per ml.

Cells were subcultured at 6- to 7-day intervals, and cultures from the 10th to 20th passage were used for the experiments.

Virus and preparation of cell-free virus. The Kawaguchi strain, which was isolated from a varicella patient in the laboratory of the Research Institute for Microbial Diseases by Takahashi on 1972, was employed for the experiments.

When more than 80% of cell sheet showed the typical cytopathic effect, the media were removed from infected cell cultures and the cells were washed twice with phosphate-buffered saline (PBS) and treated with 1% ethylenediaminetetraacetate (EDTA) in PBS to dislodge the cells from the surface of glasses. The cells were centrifuged to make pellets, and the supernatants were discarded. Suspension media consisting of PBS supplemented with 10% fetal calf serum, 50 μg of kanamycin per ml, 0.1% sodium glutaminate, and 5% (wt/wt) sucrose were added in a volume of 5 ml to each Roux bottle. Cell suspensions were sonicated with an ultrasonic disruptor (Tomy Seiko Co, Tokyo) at an intensity of 20 kcycles/s for 30 s and clarified by centrifugation at 3,000 rpm for 15 min at 4°C. Supernatants were stored at −80°C for further experiments.

Plaque assay. Monolayers of HuEF cells were grown in 60-mm glass plates and incubated at 37°C in an incubator at 5% CO_2. For infectious center assay, the dispersed cells which were already infected were suspended with maintenance medium and diluted in the same medium, and plated out in 0.2-ml amounts in 10-fold dilutions onto the monolayers in the plates. For cell-free virus titration, serial 10-fold virus dilutions (0.2 ml) in maintenance media were inoculated on HuEF cells in 60-mm plates. Virus or infected cells were adsorbed for 60 min at 37°C, and the cultures were overlaid with 5 ml of maintenance medium. Five days after infection, the cultures were fixed with a 5% solution of Formalin and stained with 0.5% methylene blue, and plaques were counted with a dissecting mi-

croscope at a ×20 magnification.

Infection of cell monolayers for virus growth. Monolayers of HuEF cells in 60-mm glass plates were inoculated with 0.3 ml of cell-free virus at a multiplicity of infection of 0.05 plaque-forming units (PFU)/cell. After 1 h of adsorption at 37°C in 5% CO_2, cells were washed with PBS three times to remove unabsorbed virus. Maintenance medium was poured onto the cells, which were reincubated at 37°C. Infected cultures were divided into two parts: one was for the detection of cell-free virus and another was for infectious center assays. At various intervals, the cells in the plates were trypsinized for the infectious center assay or treated with 1% EDTA for cell-free virus detection. For the cell-free virus detection, cells were treated with 1% EDTA to dislodge them from the glass suface and then centrifuged to pellet them. Pellets were suspended with 1 ml of suspension medium per plate and sonicated as described above. For the infectious center assay, cells were trypsinized to disperse them and then suspended with 1 ml of suspension medium per plate. Suspended cells were diluted in the same medium and plated out onto the monolayers in plates.

Preparation of antiserum. Oka strain, isolated from a varicella patient by Takahashi, was adapted to primary green monkey kidney cells and passaged seven times. Green monkey kidney cells were infected with Oka strain and cultured at 37°C. When the cytopathic effect appeared after infection, cells were washed three times with PBS, scraped off from the glass surface by a rubber policeman, and washed by centrifugation. Pelleted cells were sonicated to disrupt them and homogenized with Freund complete adjuvant, and 4 ml of the cell homogenates was injected into the green monkeys intramuscularly three times at 3-week intervals. One week after the last injection, partial bleeding was attempted. The titer of the serum used for tests was 1,024-fold by the complement fixation test. Complement fixation antibody for VZV was assayed by the microtiter method (21).

Immunofluorescent studies. HuEF cells were grown on 18-mm round cover slips resting in 60-mm glass plates. Monolayers on the cover slips were infected with VZV at multiplicilus of infection (MOIs) of ca. 0.005 PFU/cell. After an adsorption period of 1 h at 37°C, the cultures were washed with PBS and flooded with maintenance medium and reincubated at 37°C in an incubator at 5% CO_2. Some infected cover slips were cultured with the maintenance medium supplemented with 50 μg of Ara-C (Sigma Chemical Co., St. Louis, Mo.) per ml or 300 μg of PAA (ICN Pharmaceuticals, Inc., N.Y.) per ml. Infected cells grown on cover slips were harvested at various intervals for immunofluorescent tests. Cells harvested for immunofluorescent staining were washed with PBS, air dried, and fixed in a mixture of acetone and ethanol for 5 min at −20°C. They were kept at −80°C until staining. The anticomplement immunofluorescence technique was employed for staining. The fixed cover slip cultures were layered with about 0.1 ml of a mixture containing equal proportions of human complement (fresh anti-VZV antibody-negative serum) diluted 1:10 and anti-VZV serum (already absorbed with uninfected GMK cells) diluted 1:10. After incubation at 37°C for 30 min in a humidified chamber, the cover slips were washed thoroughly with PBS and layered with fluorescein isothiocyanate-conjugated goat anti-human complement C_3 serum diluted 1:20 (Hyland Laboratories, Los Angeles, Calif.). After a secondary incubation at 37°C for 30 min, the cover slips were washed with PBS, and stained cover slip preparations were mounted in a 1:9 solution of PBS and glycerin, and viewed in an Olympus fluorescence microscope.

RESULTS

VZV replication in HuEF. The infectious center assay revealed that VZV did not appear to spread to neighboring cells for 14 h postinfection (Fig. 1). About a sixfold increase in plaque-forming cells was obtained by 18 h. At this time, almost all cells contained VZV by the calculation of cell numbers. On the other hand, viral replication began between 8 and 14 h postinfection (Fig. 1).

Localization of VZV antigens in infected cells by immunofluorescent staining. Cover slip cultures fixed 2, 4, 6, 8, 14, 18, 24, and 48 h postinfection were stained with anti-VZV serum from green monkeys. Uninfected cells did not fluoresce when stained with the immune serum.

At 2 h postinfection, diffuse, weakly fluorescent material throughout the cytoplasma was observed (Fig. 2a). At 4 h postinfection, rela-

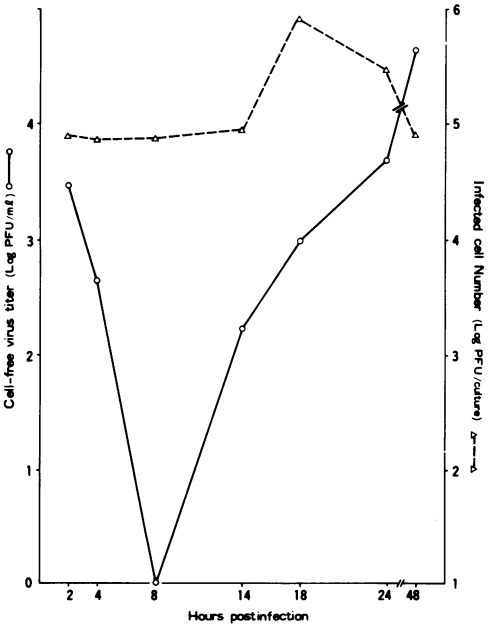

FIG. 1. *Time course of VZV infection in HuEF cells infected at a multiplicity of infection of 0.05 PFU/cell as measured by the number of infected cells* (△) *or cell-free virus* (○). *The titer of cell-free virus and infectious centers represents mean values from three experiments.*

tively bright fluorescence in the nucleus was detected (Fig. 2b). From 6 to 8 h, the fluorescence observed in the nucleus was the same as that at 4 h, but was in close association with the nuclear membrane and brighter (Fig 2c). The nuclear fluorescence got even brighter and faint fluorescence was observed in the cytoplasma by 14 h postinfection (Fig. 2d). Zoster antigens initially localized in single cells spreaded to neighboring cells by 18 h postinfection (Fig. 2e). At 24

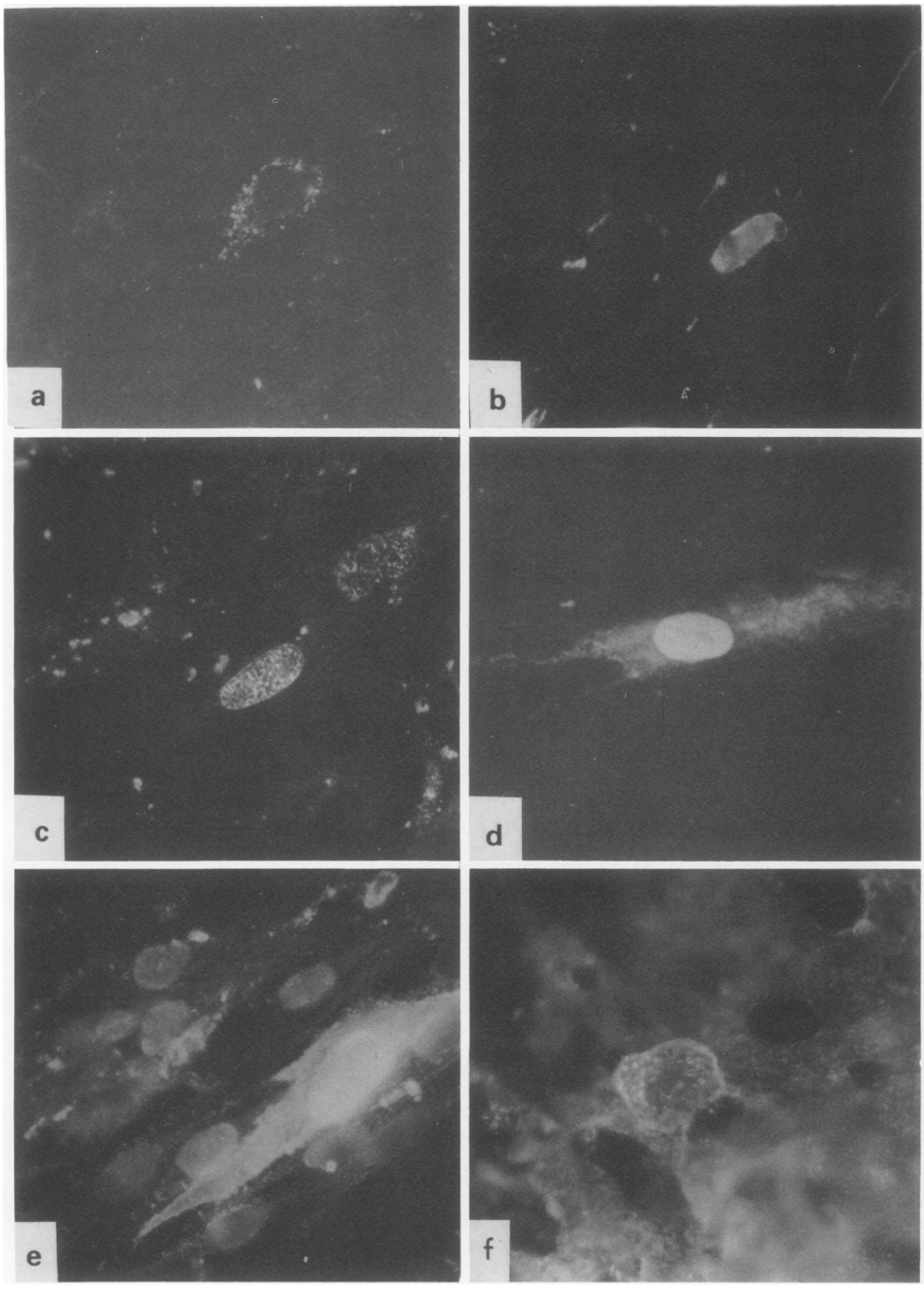

FIG. 2. *Fluorescence photomicrographs of virus-specific antigens in infected cultures at (a) 2 h, (b) 4 h, (c) 8 h, (d) 14 h, (e) 18 h, and (f) 48 h postinfection. Magnification, ×800.*

h postinfection, both bright nuclear and cytoplasmic fluorescence was observed (data not shown), and by 48 h postinfection nuclear fluorescence began to diminish and intensive perinuclear, finely granular or distributed fluorescence appeared (Fig. 2f).

Then, the effect of PAA and Ara-C on the expression of antigens was tested. PAA is known as a specific inhibitor to deoxyribonucleic acid (DNA) polymerase induced by herpesvirus infection, and Ara-C has an inhibitory action on DNA synthesis. Cover slip cultures treated with these inhibitors were also harvested at the various intervals described above. When the samples treated were compared with the nontreated ones, the inhibitors did not affect any antigen expression until 8 h postinfection (data not shown). However, very strong fluorescence remained mainly in the nucleus after 8 h and was the brightest at 48 h postinfection (Fig. 3a and b) and the antigens did not spread to neighboring cells.

DISCUSSION

In studies on the replication of VZV in human embryonic cells, Rapp and Vanderslice (11) reported that 8 to 16 h were required for the virus to infect neighboring cells. In contrast to their reports, Schmidt and Lennette (14) reported that the spread of virus to neighboring cells was demonstrable at 6 to 8 h postinfection. Both groups used the infected cells instead of cell-free virus for the infection system. However, cell-free virus is necessary for the study of replication of virus. In our report, the cell-free virus was employed for the infection, and the spreading of virus to neighboring cell started between 14 and 18 h postinfection (Fig. 1). Schmidt and Lennette (14) also used the cell-free virus for infection, and they reported that the spreading started between 24 and 48 h postinfection.

Then we attempted to assay the cell-free virus from infected cells time by time. Titers of cell-free virus started to increase between 8 to 14 h postinfection (Fig. 1). In contrast to our data, Schmidt and Lennette (14) reported that titers of cell-free virus started to increase between 24 and 48 h postinfection and reached the maximum at 48 h after infection. However, it is difficult to compare these data because the latter started to make plots at 24 h postinfection.

Herpes simplex virus replicated and released progeny by 8 h postinfection, whereas human cytomegalovirus required 4 days (16), when cell-free virus was added to the cultures. Our data suggest that 8 to 14 h would be required for maturation and that it would take 4 more h to spread to neighboring cells (Fig. 1). So the maturation of VZV quite resembled that of herpes simplex virus.

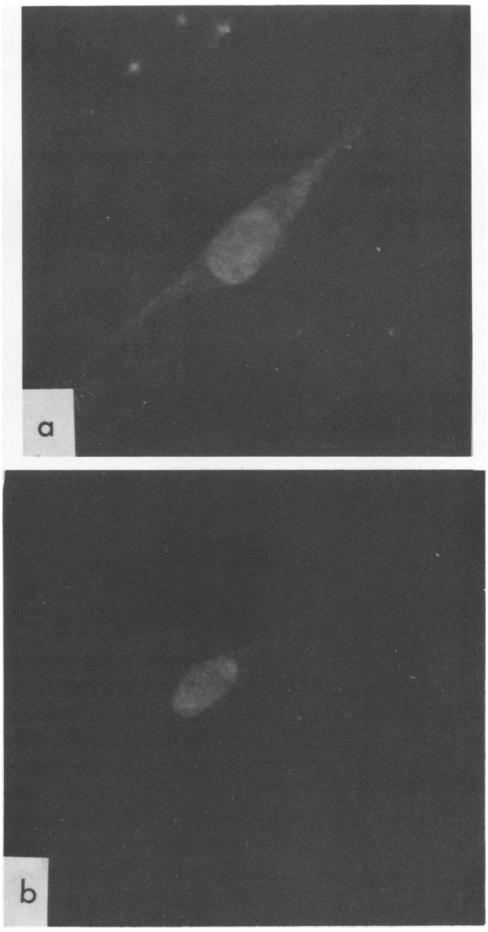

FIG. 3. *Fluorescence photomicrographs of virus-specific antigens in infected cultures treated with (a) Ara-C (50 μg/ml) and (b) PAA (300 μg/ml) and fixed at 48 h postinfection. Magnification, ×800.*

Weller and Coons (20) first described the localization of VZV antigens in human cells. Slotnick and Rosanoff (15) reported that VZV antigen, detected by the immunofluorescent antibody test was in the cytoplasma at the border of the nucleus, gradually accumulated in the perinuclear region, and finally concentrated in the cytoplasma. They also reported that nuclear antigen was not observed before 72 h postinfection. Rapp and Vanderslice (11) described that VZV antigen was first detected by 16 h postinfection. Koller et al. (5) also reported that viral antigen was first detectable about 10 h postinfection in the nucleus. Between 24 and 48 h, nuclear fluorescence increased and cytoplasmic fluorescence appeared. After 72 h, the antigens gradually disappeared from the nucleus. The present studies have shown that the first ap-

pearance of the viral antigen in cells infected with VZV was demonstrable in the cytoplasma in the form of diffuse fluorescence at 2 h postinfection (Fig. 2a). By 4 h, the diffuse fluorescence was observed in the nucleus (Fig. 2b), and by 6 to 8 h, the fluorescence of the nuclear membrane region was strong (Fig. 2c). The cytoplasmic and nuclear membrane strongly fluoresced by 24 h postinfection. In case of herpes simplex virus infection, diffuse weakly fluorescent material throughout the cytoplasma was observed by 2 h postinfection, and by 3 to 6 h, the nuclei were filled with intensive fluorescence (13). This fact also resembles the case of VZV.

So far, different immunofluorescence studies on VZV-infected cells have been difficult to reconcile for two reasons. One is that antisera with very different qualities have been used. Almost all investigators have used convalescent sera from patients. On the other hand, we used sera from green monkeys artificially highly immunized with Freund complete adjuvant, and so it seems that our sera would contain multiple antibodies, including antibodies against early antigens. In addition, the method of staining used in previous studies differs from that used in ours. The anticomplement immunofluorescent test was employed in our experiments. This method is a modification of the indirect fluorescent antibody technique. The advantage of this method over the indirect method used in previous reports is that nonspecific cytoplasmic fluorescence seen in the indirect test can be eliminated. Furthermore, this method has been shown to be highly sensitive when it has been employed for Epstein-Barr virus (12) and cytomegalovirus (1, 10).

The inhibitory effect of PAA on the replication of VZV has been reported by May et al. (8). PAA appears to act by inhibiting the VZV-induced DNA polymerase and blocking viral DNA synthesis (6, 9) as with other herpesviruses (4, 7). Ara-C does not affect the production of early antigens but that of late antigens in herpesvirus-infected cells (1–3, 18, 19). A nuclear antigen has previously been demonstrated by immunofluorescence tests in cytomegalovirus and herpes simplex virus-infected and Ara-C-treated cells (2, 3, 17). With VZV, it would also be possible to distinguish between early antigens and late proteins. So, these two inhibitors were applied to the VZV-infected cells, and the effect on the antigen expression was tested. Fluorescence was seen mainly in the nuclei (Fig. 3a and 3b). This feature resembles that of infected and nontreated cells at 4 h. Those results suggest that the antigens detected in nuclei in infected cells cultured with inhibitors would be early antigens, which resemble the early antigen reported in herpesvirus-infected cells.

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