JOURNAL OF VIROLOGY, Nov. 1972, p. 995-1001
Vol. 10, No. 5
Printed in U.S.A.
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Latency of Human Measles Virus in Hamster Cells

PAUL KNIGHT, RONALD DUFF, AND FRED RAPP

Department of Microbiology, College of Medicine, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

Received for publication 21 August 1972

A latent system employing measles virus (Schwarz strain) was developed in hamster embryo fibroblasts (HEF). Measles virus-specific antigen was detected by immunofluorescence in 30 to 50% of HEF cells, and these cells released infectious virus when co-cultivated with a susceptible monkey cell line, BSC-1 cells. No infectious virus could be detected in the cells when measures were taken to exclude passage of viable latent cells onto the indicator BSC-1 cells. Infectious center assays demonstrated that about 1 in 10 of the latently infected cells in the population could release infectious virus. Infectious virus appeared within 6 hr after co-cultivation of the HEF cells with BSC-1 cells, as compared to 24 hr required for normal replication of measles virus in the BSC-1 cells. Furthermore, labeling of progeny virus ribonucleic acid (RNA) by using tritiated uridine, and inhibition of RNA or protein synthesis by 5-azacytidine or cycloheximide suggested that neither additional RNA nor protein synthesis is required after co-cultivation of the cells to effect early virus release. It can therefore be postulated that there is a block at a late step in virus replication in the latently infected hamster cells. The most obvious site would concern maturation of infectious virions at the cell membrane.

Measles virus was implicated in diseases which may result from the latency of the virus within the infected cell. Notable among these diseases are subacute sclerosing panencephalitis (SSPE) (5) and multiple sclerosis (3). Moreover, in vitro cell culture techniques have shown that measles virus can be carried in cells for prolonged periods without release of infectious virus (13).

In vitro as well as in vivo studies have shown tubules containing measles virus nucleoprotein and virus-specific antigens (5, 9, 13) in cells not releasing infectious virus. In SSPE, an agent presumably identical or very similar to measles virus has been isolated by co-cultivation of SSPE cells with another cell line permissive for measles virus replication (1, 4). Recently, we have shown that certain strains of measles virus cause acute encephalitis when injected intracerebrally into newborn hamsters (16). Under appropriate conditions, some of these animals yielded evidence of long-term latency of the virus after inoculation as newborns (17). It therefore seemed of interest to determine whether a comparable system could be developed by employing cultured cells. This would enable study of molecular events responsible for maintaining latency of this virus in the hamster cells.

Several mechanisms by which measles virus remains latent have been proposed. These include defective particle inhibition of virus replication (7, 13) and resistance of the host cell to release of infectious units (14). This report describes a system of latency with measles virus established in vitro and investigates the mechanisms which are responsible for the maintenance of this latency.

MATERIALS AND METHODS

Virus. The Schwarz measles virus, vaccine lot no. 94217, was obtained from Merck, Sharp & Dohme. This virus was passaged eight times in BSC-1 cells. Virus stocks were grown in monolayers in 8-oz (0.24-liter) bottles. After 96 hr, cells were scraped off the glass surface, disrupted by sonic oscillation, and clarified by low-speed centrifugation. These stocks were quick-frozen in a dry ice-alcohol bath and stored at −90 C.

Cells. Hamster embryo fibroblast (HEF) cells were prepared from 13-day-old Syrian hamster embryos. Decapitated embryos from a single litter were trypsinized and grown at 37 C in 8-oz prescription bottles. BSC-1 cells obtained from R. Dulbecco at the Salk Institute were also grown in 8-oz bottles at 37 C.

All cells were grown in Eagle basal medium containing 10% fetal calf serum, 10% Tryptose phosphate broth, and 0.08% $NaHCO_3$. After infection with virus, HEF cells were grown in low calcium Eagle medium with the same additives as above.

Plaque assay. BSC-1 cell monolayers, grown in 60-mm Falcon plastic petri dishes, were inoculated with 0.1 ml of the measles virus suspension to be assayed. The virus inoculum was diluted with 0.4 ml

of tris(hydroxymethyl)aminomethane (Tris)-buffered saline (*p*H 7.4, 0.025 M) after addition to the cultures, to prevent drying of the cells. After virus adsorption for 2 hr at room temperature, the BSC-1 cell monolayers were overlaid with Eagle basal medium containing 10% fetal calf serum, 1% agar, and 0.23% $NaHCO_3$. The infected cultures were kept in a 5% CO_2 incubator at 37 C for 7 days, after which time 2 ml of Tris-buffered saline (*p*H 7.4) containing a 1:20,000 dilution of neutral red was added. Plaques were counted 8 days after initial virus adsorption.

Virus neutralization tests. Virus (0.5 ml) was incubated at 37 C for 40 min in 0.5 ml of measles-specific antiserum serially diluted in Tris-buffered saline (*p*H 7.4). The measles-specific antisera were prepared in hamsters. Remaining infectious virus was determined by the plaque assay described above.

Immunofluorescence techniques. Virus-infected and uninfected control cells were grown on 15-mm cover slips. After 24 hr of growth, the cultures were washed three times in warm Tris-buffered saline (*p*H 7.4) and air dried, followed by fixation for 3 min in acetone. Sera from patients with SSPE (diluted 1:100) obtained from P. A. Fuccillo at the National Institutes of Health, or measles-immune serum (undiluted) from hamsters were absorbed onto the cells on each cover slip for 30 min at 37 C. After being washed three times in warm Tris-buffered saline (*p*H 7.4), the cells were exposed to anti-human (or anti-hamster) gamma globulin labeled with fluorescein isothiocyanate for 30 min at 37 C. The cells were viewed by using a Zeiss fluorescence microscope.

Fusion of cells by using inactivated Sendai virus. BSC-1 (10^7 cells) and latently infected HEF (5×10^6 cells) were mixed and pelleted by centrifugation at 1,000 rev/min. Sendai virus was inactivated by ultraviolet irradiation for 8 min with 42.5 erg per sec per cm^2. One milliliter of the inactivated Sendai virus containing 800 hemagglutination units was added slowly to the cell mixture. After cooling for 10 min on ice, the cells were shaken for 20 min at 37 C, washed once with Eagle medium, centrifuged at 1,000 rev/min, and placed in 8-oz bottles with low calcium Eagle medium (0.1 mM $CaCl_2$) plus 10% fetal calf serum, 10% Tryptose phosphate broth, and 0.08% $NaHCO_3$.

Labeling of virus ribonucleic acid (RNA). All cells were placed in Eagle medium with 10% dialyzed fetal calf serum 48 hr before labeling and during the time of labeling. After exposure to measles virus (by cocultivation or adsorption of stock virus), 10 μCi of [^{3}*H-5*]uridine per ml (New England Nuclear Co., Boston, Mass.) was added. Chases were performed by using 50 μg of cold uridine per ml. After harvesting 48 hr postinfection, the virus was exposed to 50 μg of pancreatic ribonuclease per ml (Sigma Chemical Co., St. Louis, Mo.) for 4 hr at 4 C. The labeled virus was then precipitated with 50% saturated $(NH_4)_2SO_4$, redissolved in Tris-buffered saline (*p*H 7.4) plus 0.001 M ethylenediaminotetraacetic acid, and purified on a 15 to 60% sucrose gradient in a Spinco SW41 rotor at 36,000 rev/min for 2 hr. The band containing infectious virus was removed, and trichloroacetic acid-precipitable counts were monitored in a Beckman LS 250 liquid scintillation counter.

Inhibitors. The compounds, 5-azacytidine (Calbiochem, Los Angeles, Calif.) and cycloheximide (Sigma Chemical Co., St. Louis, Mo.), were dissolved in Eagle basal medium at a concentration of 500 μg/ml and 10 mg/ml, respectively, quick-frozen in a dry ice-alcohol bath, and stored at -10 C. When used, they were thawed and diluted so the final concentrations in the growth medium were 10 μg of cycloheximide per ml and 50 μg of 5-azacytidine per ml.

RESULTS

Conditions necessary to establish measles virus latency. Primary HEF were inoculated with the Schwarz vaccine strain of measles virus in suspension with shaking at a multiplicity of 0.01 plaque-forming units (PFU) of virus per cell for 3 hr at room temperature. The cells were seeded into 60-mm petri dishes at a density of 2.5×10^5 cells per plate. After 21 days, the cells were trypsinized and passed into 8-oz bottles. After five passages, several of the cultures infected with measles virus developed multinucleated giant cells, extensive virus cytopathology (CPE), and produced infectious virus. These were discarded.

Between passages 8 and 12, measles-infected HEF cells without visible virus CPE were assayed for infectious virus. Several methods to release infectious measles virus from infected cells were used (Table 1). These included freezing and thawing, sonic disruption followed by freezing and thawing, filtration of supernatant fluids through a Millipore AP-200 prefilter (previously shown to prevent passage of cells but not of virus), and sonic disruption followed by filtration of the sonically treated cells. No infectious virus was detected when these conditions for harvesting excluded the possibility of passing viable cells on to the assay cultures of BSC-1 cells.

After 20 passages, the cell cultures began producing infectious virus at a rate of 1 PFU per 20,000 cells when grown at 37 C. This could be enhanced to 1 PFU per 200 cells by growing the cells at 33 C. Release of infectious virus was eliminated by incubating the cells at 39 C. Therefore, all tests after passage 20 were carried out at this temperature.

Detection of virus-specific antigens in cells latently infected with measles virus. It was previously shown that cell lines carrying latent measles virus contain measles virus-specific antigens in the cytoplasm and in the nucleus (13). To test this in our system, cells were grown on cover slips and stained with measles virus-specific antisera by utilizing the indirect immunofluorescence test. There was perinuclear fluorescent staining in approximately 30 to 50% of the cells in the culture (Fig. 1). The antigen was seen less often in the nucleus or in cytoplasmic processes (Fig. 2). The

TABLE 1. *Attempts to isolate infectious virus from HEF latently infected with measles virus*[a]

Cell history	Measles virus isolation				
Passage no. after exposure to Schwarz measles virus	Frozen and thawed twice	Sonically treated and filtered through a Millipore AP-200 prefilter	Sonically treated, frozen, and thawed	Filtration of the supernatant fluids through a Millipore AP-200 prefilter	Co-cultivation with BSC-1 cells
12	<2 PFU/ml	ND[b]	<2 PFU/ml	ND	+[c]
12	<2 PFU/ml	ND	<2 PFU/ml	ND	+
14	ND	<2 PFU/ml	<2 PFU/ml	<2 PFU/ml	10^6 PFU/ml
14	ND	<2 PFU/ml	<2 PFU/ml	<2 PFU/ml	5×10^5 PFU/ml
16	ND	ND	<2 PFU/ml	ND	+
18	ND	ND	<2 PFU/ml	ND	+
20	ND	9×10^1 PFU/ml	5×10^1 PFU/ml	<2 PFU/ml	5×10^6 PFU/ml

[a] HEF latently carrying measles virus were harvested by different techniques and assayed on BSC-1 cells for infectious virus.
[b] Not done.
[c] 4+ cytopathic effect not measured by plaque assay.

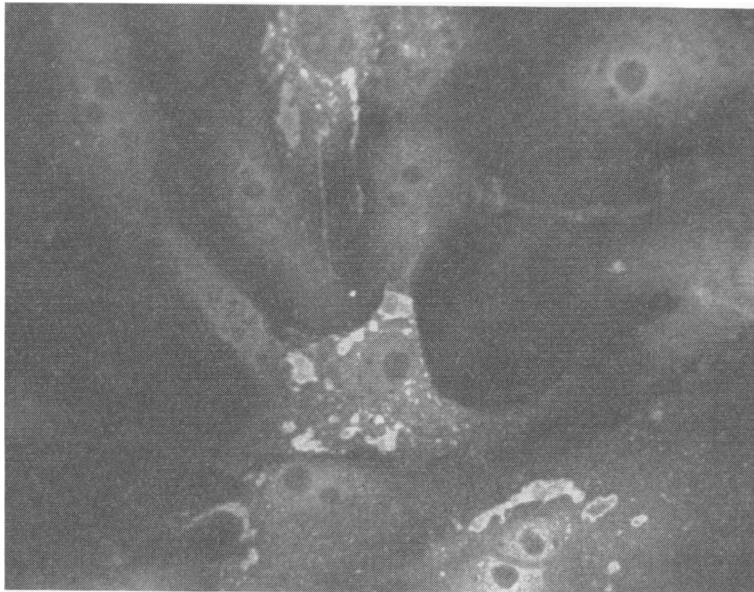

FIG. 1. *Immunofluorescence photomicrograph of measles virus-specific antigens in the cytoplasm of HEF cells latently infected with measles virus (×360).*

antigen was generally observed as irregular aggregates in the cytoplasm. Many cells with an apparently normal morphology, including cells in mitosis, contained detectable measles virus antigens.

Co-cultivation of HEF cells carrying latent virus with BSC-1 cells. It has been shown in SSPE that an infectious measles-like virus can be rescued by co-cultivation of biopsy cells with a normally susceptible cell line (3). We therefore utilized BSC-1 cells as the susceptible cell line, to rescue virus from the infected HEF carrier line

Co-cultivation of 10^6 BSC-1 cells with 10^6 cells carrying latent measles virus produced measles virus CPE (large syncytia) within 6 hr after the two cell types came in close contact. Extensive CPE occurred within 24 hr with a release of infectious virus (about 10^6 PFU of culture fluid per ml). All BSC-1 cells became detached from the surface by 48 hr postfusion. The HEF cells were not destroyed and continued to carry the virus, which could be demonstrated by the addition of more BSC-1 cells, again inducing the release of infectious measles virus. The agent

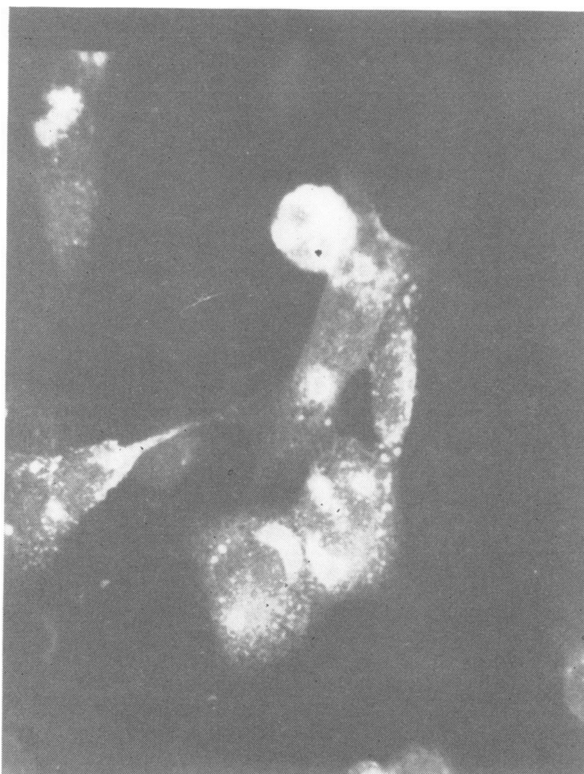

FIG. 2. *Immunofluorescence photomicrograph of measles virus-specific antigens in the nucleus and in cytoplasmic process of HEF cells carrying latent measles virus* (×*360*).

released by co-cultivation was neutralized by measles-specific antisera and therefore appeared identical to the original stock of measles virus used to infect and establish the latent system in HEF.

The co-cultivation technique described above was also utilized to demonstrate how many cells in the population were releasing virus upon contact with BSC-1 cells. Serial tenfold dilutions of the cells carrying latent virus were added to monolayers of BSC-1 cells in 60-mm plates. Each monolayer of BSC-1 cells contained approximately 10^6 cells per plate. The HEF cells were allowed to attach to the BSC-1 monolayer, and the cultures were then overlaid with medium containing agar. Plaques appeared 48 hr after infection, two days earlier than the appearance of plaques in BSC-1 cells infected with measles virus not associated to HEF cells. One in 10 of the HEF cells released infectious virus after co-cultivation with BSC-1 cells.

Replication of measles virus after co-cultivation of hamster and BSC-1 cells. In the previous experiments, three observations were consistent with the hypothesis that the BSC-1 cells caused the maturation and release of partially preformed virus already present in the HEF cells latently infected with measles virus. (i) measles CPE (giant cell formation) appeared soon after the two cell types came within close contact. (ii) Plaques appeared in the infectious center assay 2 days earlier than they normally do in the same BSC-1 cells when infected with measles virus under identical conditions. (iii) Virus titers obtained by co-cultivation were greater than had been obtained by growth in BSC-1 cells alone, suggesting a burst or release of virus that had accumulated due to a block late in the virus growth cycle.

To test this hypothesis, the time of appearance of infectious virus in the co-cultivation system was compared with the appearance of virus in measles virus-infected BSC-1 cells. Schwarz measles virus progeny did not appear earlier than 24 hr in BSC-1 cells and reached maximal titers at 36 to 48 hr. This agrees with results obtained earlier in our laboratory (12) and by other investigators (6). Co-cultivation of BSC-1 cells with latently infected HEF cells resulted in the appearance of infectious virus 8 hr after co-culti-

vation, with maximal levels occurring at approximately 16 to 24 hr (Fig. 3).

The time of the first appearance of measles virus can be reduced by fusion of 5×10^6 HEF with 10^6 BSC-1 cells by using inactivated Sendai virus; under these conditions, infectious measles virus appeared 1 to 2 hr after fusion. However, the titers obtained by using Sendai virus were not as high as those obtained without Sendai virus (Fig. 3).

These co-cultivation experiments were repeated with rabbit kidney cells, a cell line relatively resistant to measles infection and uninfected HEF cells. Neither procedure induced the release of infectious virus.

To determine if progeny virus RNA is formed prior to virus release from latently infected HEF cells by co-cultivation with BSC-1 cells, labeling experiments were carried out with tritiated uridine. ^{3}H-uridine (10 μCi/ml) was added to the medium for 24 hr after co-cultivation with BSC-1 cells. The results were compared to a 24-hr pulse of ^{3}H-uridine in the permissive BSC-1 cells which had been infected with measles virus (Table 2).

FIG. 3. *Comparative replication of measles virus in BSC-1 cells under different conditions. BSC-1 cells were infected with cell-free measles virus (—□—), co-cultivated with HEF cells latently containing measles virus (—●—), and fused with HEF cells latently containing measles virus by using inactivated Sendai virus to promote cell fusion (—○—).*

TABLE 2. *Incorporation of labeled ^{3}H-uridine into measles virus RNA*[a]

Mode of infection	Counts per min per 10^3 PFU
Adsorbed virus	2,164
Co-cultivation	398

[a] Schwarz measles virus, 10^5 PFU, or 10^5 infectious units of HEF latently infected with measles as determined by infectious center assay were placed on BSC-1 cells in the presence of 10 μCi of [^3H-5]uridine per ml for 20 hr. After isopycnic banding on a 15 to 60% sucrose gradient, counts per minute per PFU were determined.

The measles virus released from infected BSC-1 cells incorporated three times more label per infectious virus than did the measles virus released after co-cultivation of the two cell types.

Latently infected HEF cells were pulse labeled for 1 hr with ^{3}H-uridine after co-cultivation with BSC-1 cells. Samples were collected at 1-hr intervals during the first 10 hr after infection. After 1 hr of exposure to ^{3}H-uridine, cultures were chased with 50 μg of cold uridine per ml. Results demonstrated that at no period of time is ^{3}H-uridine incorporated into progeny virus RNA. However, ^{3}H-uridine was incorporated into progeny virus RNA between 4 and 7 hr after the infection of BSC-1 cells with free measles virus (Fig. 4). These observations are consistent with the hypothesis that the RNA of progeny virus is preformed prior to release by co-cultivation.

Effect of RNA and deoxyribonucleic acid inhibitors on virus release by co-cultivation. An RNA analogue, 5-azacytidine, has been shown to mutagenize and inactivate RNA viruses. It was postulated that this occurs by incorporation of the RNA analogue into the virus genome (2). In a preliminary experiment, the release of infectious measles virus was completely inhibited by 50 μg of 5-azacytidine per ml when the mutagen was added within 5 to 6 hr after absorption of measles virus to BSC-1 cells. In the absence of inhibitor, 5×10^5 PFU of virus per ml were released. However, no significant inhibition in the release of infectious virus was observed when 50 μg of 5-azacytidine per ml was added to the medium at the time of co-cultivation (Fig. 5). This mutagen had little effect on the release of infectious virus, even when left on the HEF and BSC-1 mixture for up to 12 hr. However, a decrease of over half a log in the release of infectious virus was noted if the mutagen remained for 16 hr after co-cultivation. These results agree with the ^{3}H-uridine labeling experiment which suggested that virus

progeny RNA is preformed and is being carried in the latently infected HEF cells.

It was previously demonstrated in cells persistently infected with mumps virus that protein synthesis did not appear to be required for the release of virus from latently infected cells (8). In a preliminary experiment, cycloheximide (10 μg/ml) inhibited the appearance of infectious virus from BSC-1 cells when added to the medium any time prior to 24 hr after infection. However, the addition of 10 μg of cycloheximide per ml to the medium at the time of co-cultivation did not inhibit the appearance of virus up to 8 hr after co-cultivation (Fig. 5). After 8 hr the virus yield began to decrease, and after 16 hr post co-cultivation, the virus yield decreased 2.5 logs less than the controls. No difference in measles CPE (giant cell formation) was noticed in the co-cultivation cultures, with or without the inhibitor. These data suggest that, up to 8 hr after co-cultivation, little, if any, protein synthesis is required for the release of infectious virus after co-cultivation of latently infected HEF and susceptible BSC-1 cells.

FIG. 4. *Requirement for new virus RNA synthesis for production of infectious virus from 5 × 10^6 BSC-1 cells co-cultivated with 10^5 infective units of HEF cells latently infected with measles virus (—O—) or infected with 10^5 infectious measles virus particles (—●—). Each culture was exposed to [^{3}H-5]uridine for 1 hr with samples collected hourly for first 10 hr after infection of BSC-1 cells. Virus was banded in a 15 to 60% sucrose gradient and assayed in a scintillation counter for ^{3}H counts/min.*

FIG. 5. *Effect of 50 μg of 5-azacytidine (—●—) per ml or 10 μg of cycloheximide (—O—) per ml on release of measles virus after co-cultivation of BSC-1 cells with latently infected HEF cells. Co-cultivation involved 5 × 10^6 BSC-1 cells and 10^5 infectious units of latently infected HEF cells. Number of cellular infectious units was determined by infectious center assay. Cycloheximide or 5-azacytidine was added at time of co-cultivation and remained in culture medium until samples were collected for infectious virus assay. Results obtained by using inhibitors of protein and RNA synthesis are compared to normal release of infectious virus after co-cultivation (—▲—).*

DISCUSSION

Measles virus produces two types of infection in primary HEF. The first is a lytic infection in which most of the cells are destroyed, with subsequent release of large quantities of virus. The second type is characterized by a chronic or latent infection where very little, if any, infectious virus is released. The chronic infection may occur by propagation of the few remaining cells after a lytic infection, or may exist from the start of infection with no measurable lytic activity in the cell culture.

Measles virus which remains latent in HEF can be induced by co-cultivation with BSC-1 cells,

a virus-permissive cell line. It appears that the process by which the virus is released is mediated by fusion of the two cell types, since extensive giant cell formation occurs after the two cell types come into intimate contact, and the time required for infectious virus release can be shortened if the two cell types are fused by using inactivated Sendai virus. However, the possibility of a transferable factor from BSC-1 cells to the HEF cells has not yet been ruled out.

It is consistent with the experimental data that the BSC-1 cells can induce the release of infectious measles virus which has proceeded to a late point in the virus replication cycle within the HEF cells. The quick release of large quantities of virus upon contact between the two cell types, the presence of large amounts of measles virus-specific antigens in the cells, the existence of preformed progeny RNA, and the need for little or no new protein synthesis for early release after co-cultivation suggest that a late maturation step (perhaps involved in budding) is the site of the block in synthesis of infectious virus, as has been demonstrated in persistently infected cultures of mumps virus (8).

This latency of measles virus in HEF may be accompanied by the selection of virus which is temperature sensitive. This has been demonstrated in the RNA phage system (15) and with a related paramyxovirus, Newcastle disease virus (10). Experiments are under way to test this hypothesis.

It has also been proposed by Rustigian (14) that latency may occur by the selection of cells that are more resistant to the infecting virus. This may be the case in our latently infected HEF, since the virus obtained by co-cultivation behaves normally in BSC-1 cells and can cause a lytic infection in primary HEF which have not been previously exposed to measles virus.

Recently, it was shown by electron microscopy that virus tubules in hamster dorsal-root ganglion cells, chronically infected with measles virus, appear strikingly similar to those taken of cells obtained by biopsy from SSPE patients (11). Similarly, the appearance of the antigen detected by immunofluorescence in our latent cells and in SSPE biopsied cells was nearly identical. Possibly, a similar mechanism exists between latency of measles virus in our system and that of latent measles virus in cells from patients with SSPE.

ACKNOWLEDGMENTS

This study was conducted under Public Health Service contract no. 70-2024 within the Special Virus Cancer Program of the National Cancer Institute, and by Public Health Service research grant CA-11647 from the National Cancer Institute. The work was also supported by grant no. 701-A-1 from the National Multiple Sclerosis Society.

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