Characterization of the Growth of Herpes Simplex Virus in Human Lymphoid Cells

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Herpes simplex virus was grown in a 6-liter suspended culture of an atypical permanent human lymphoid cell line, Roswell Park Memorial Institute no. 8226. The kinetics of virus replication were determined by counting viruses by electron microscopy, plaque formation, and tissue culture infectivity. Deoxyribonucleic acid-dependent deoxyribonucleic acid polymerase activity was determined during the course of infection. Electron microscopy studies substantiated the kinetics of the virus infection in lymphoid cells.

There is increasing evidence that viruses are the cause of some types of human malignancies (28). Herpstype viruses have been found to be the causative agents of several animal tumors (7, 15, 18, 39). Indirect evidence indicates that herpes simplex virus (HSV) may cause cancer in man (32). HSV was found associated with cervical carcinoma (29, 30). Therefore, it is important to study HSV replication in various human cell types. It has been studied extensively by electron microscopy in small-scale monolayer cultures of several types of cells (6, 13, 23, 25, 36). In 1957 Kaplan studied the growth of HSV in a small-scale suspended culture of rabbit kidney cells (10).

The aim of this work was to characterize more fully the growth of HSV in a large-scale human lymphoid cell culture. Although human lymphoid cells can be grown in roller bottle cultures such as those used by Roizman and Spear (33), they do not attach to glass; therefore, a large-scale culturing method was developed which allowed for maximum virus yields in lymphoid cells growing in suspension culture.

During the course of viral infection, several culture parameters were measured and compared with those of uninfected cultures. The condition of the cells during infection was evaluated by taking total cell counts, determining cell viability by the trypan blue exclusion method (17), and by examining with the electron microscope. The replication of the virus was followed by a microtitration assay, (31), plaque assay (34), and particle counts by electron microscopy (19).

In the last two years, great attention has been given to deoxyribonucleic acid (DNA) polymerases in normal and malignant cells infected and noninfected with ribonucleic acid (RNA) oncogenic viruses (3, 8, 37). DNA-dependent DNA polymerase (DdP) and especially RNA-dependent DNA polymerase (RdP) have been intensively investigated for possible association of a virus with malignancy (8). Therefore, the levels of DNA polymerase were also followed during the course of infection.

MATERIALS AND METHODS

Lymphoid cell line. The RPMI cell line 8226 used for virus propagation was derived from a multiple myeloma patient. Its morphology and some of its biological characteristics have been described by Matsuoka et al. (16). Repeated electron microscopy and immunofluorescence investigations indicated that herpeslike viruses (Epstein-Barr virus) could not be detected in these cells (22).

HEL fibroblast cell line. A diploid fibroblast cell line was derived as a monolayer from human embryonic lung (HEL), and used for HSV stock preparation and for HSV infectivity determinations.

HSV. HSV type 1, after 80 passages in HEL cells, was used in this work. This strain was isolated at the Institute from a patient with recurrent herpetic skin disease.

RPMI 1701 medium. This medium is similar to RPMI medium 1640 (20) except for the following changes. (i) Calcium nitrate and magnesium sulfate were reduced to 50 mg/liter each. (ii) The following ingredients were added (mg/liter): oxalic acid, 150; sodium pyruvate, 50; sodium carboxymethyl cellulose, 1,000 (Edifás B-50, 15 centipoises, Imperial Chemical); N-2-hydroxyethyl-piperazine-N'-2'-ethanesulfonic acid (HEPES), 7,000; and insulin, 200 units/liter. The final pH of the medium was 6.9 to 7.1.

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This medium supplemented with 2% (v/v) fetal bovine serum (FBS) was used for growth of RPMI cell line 8226. The same medium supplemented with 1% FBS was used for HSV propagation in suspended cultures.

**Eagle's medium.** Eagle's basal medium in which the amino acids and vitamins were in 3x concentration (Associated Biomedic Systems, Inc., Buffalo, N.Y.) supplemented with 10% FBS was used for growth of HEL cells. The same medium was used with 1% FBS for virus production in HEL cells.

All media contained 100 units of sodium G-penicillin per ml and 50 μg of streptomycin per ml.

**Culture vessels.** HEL cells were grown in 16-oz (0.473 liter) prescription bottles. RPMI cell line 8226 was grown in Spinner flasks and fermentors. Cultures of 150 ml were maintained in Spinner flasks with a 500-ml total capacity. The flasks were modified as described by Moore et al. (21). Bench scale cell cultivation was done in 14-liter fermentors (MicroFerm, New Brunswick Scientific Co., Inc.) with a working volume of about 6 liters. These fermentors contained four baffles and one impeller mounted at the 0-liter level. Agitation in cultures was kept at 100 rev per min.

**Cell counts.** Cell counts and cell viability were measured by the trypan blue exclusion method (17). The condition of the cells was also checked in sections by electron microscopy.

**Preparation of culture sample.** Ten-milliliter samples of the HSV-infected cultures were taken at the times indicated below and were separated into cells and supernatant fluid by centrifugation at 500 × g for 3 min. Virus determinations and DNA polymerase assays were investigated in the culture supernatant fluid as well as in cell lysates produced by freezing and thawing. For electron microscopy, 35-ml samples were centrifuged at 500 × g for 3 min, and the cells were immediately fixed and prepared for thin sectioning as described by Anderson (2).

**Virus determination.** Three methods of virus quantitation were used. (i) By using the microtitration assay 3, the median tissue culture infective dose (TCID₅₀) in HEL cells was determined. Serial 10-fold dilutions of the virus sample were prepared in 3 × Eagle's medium with 1% FBS. To each virus dilution was added an equal volume of freshly trypsinized HEL cells having 2 × 10⁵ to 3 × 10⁶ cells/ml in 3 × Eagle's medium with 10% FBS. Immediately thereafter, 0.2 ml of each of the virus dilutions was placed into 5 to 10 cups of a Microtest II tissue culture plate. These assay plates were incubated for 3 days in a CO₂ incubator and the cytopathogenic effect was observed. The TCID₅₀ per ml was calculated by the method of Reed and Muench (31). (ii) With the plaque assay method, a modification of Russell's (34) plaque assay was used. Serial 10-fold dilutions of test samples in 3 × Eagle's medium with 1% FBS were prepared and assayed in triplicate as follows. A 0.2-ml amount of each virus dilution was placed onto 1-day grown HEL monolayers in Petri dishes (60 by 15 mm) from which the growth media had been removed. After 1 hr at room temperature for virus adsorption, the monolayers were overlaid with 5 ml of 3 × Eagle's medium with 1% each of FBS and an immune human serum. After incubation in a CO₂ incubator for 3 days, the monolayers were stained with 0.03% crystal violet diluted 1:1 with 10% Formalin. HSV plaques were counted and results were expressed as plaque-forming units (PFU) per ml of the original culture supernatant fluid or cell lysate. (iii) To determine virus particle counts by electron microscopy, 5-ml samples of culture supernatant fluids and cell lysates produced by freezing and thawing were centrifuged at 60,000 rev/min (4 C) for 2 hr. The sedimented materials were resuspended by mild sonication treatment for 1 min in 0.1 ml of phosphate-buffered sucrose (50 mm potassium phosphate and 0.3 mm sucrose, pH 7.2). Polyethylene latex spheres (0.088 μm d, Dow Chemical Co.) were added to the buffered suspension. The viruslike particles (VLP) were counted by electron microscopy after negatively staining with 1% potassium phosphotungstate solution, pH 7.2. The method used was similar to that of Monroe and Brandt (19). Results are expressed as VLP per milliliter of the original culture supernatant fluid or cell lysate.

**Electron microscopy.** Sections and negatively stained samples were examined in a Siemens Elmiskop 1A electron microscope using a 20-μm objective aperture and operating at 80 kv.

The cells in sections were counted and the percentage of disintegrating cells (DC) was calculated for 100 cells. A cell was considered to be disintegrating if its cytoplasmic membrane was broken. The percentage of total cells with associated virus (attached to the cell membrane, within the cytoplasm or inside the nucleus) and the percentage of DC with virus within the nucleus was also calculated.

**DNA polymerase measurements.** RdP was determined by the methods of Gallo et al. (8) and Scolnick et al. (35). DdP determination was based on the incorporation of thymidine triphosphate labeled with tritium (18.3 Ci/m mole; New England Nuclear Corp.) into deoxyribonucleic acid. HSV-infected cells were washed twice with PBS and in a buffer solution which contained 0.02 m tris(hydroxymethyl) aminomethane-hydrochloride, 0.06 m magnesium acetate and 0.0001 m disodium salt of ethylenediaminetetraacetic acid (EDTA), pH 8.3. Samples were resuspended in the same buffer without EDTA. Then the samples were pottered and sonically treated at the highest power. The cell lysate was centrifuged at 30,000 × g for 30 min at 4 C. Enzyme activity was then determined in the 30,000 × g supernatant fluid. DdP enzyme activity was also determined directly in the culture supernatant fluid. Enzyme activity is expressed as 1H counts per minute per microgram of protein. DNA polymerases were measured both in noninfected RPMI 8226 cells and in HSV-infected RPMI 8226 cells.

**Protein determinations.** Protein was determined by the method of Lowry et al. (14). Nucleic acids were measured as described by Burton (5).

**RESULTS**

RPMI cell line 8226 cultures were maintained in the exponential growth phase by
daily feeding with 10% (v/v) fresh medium. After infection with virus, the cultures were fed with RPMI 1701 medium supplemented with 1% FBS. In Fig. 1 the steps in the virus propagation are given. All cultures of cells and virus were incubated at 36 to 37°C.

The following experiments were carried out in Spinner cultures to determine the optimal conditions for virus replication in large-scale suspension cultures.

**Effect of different serum concentrations on virus production.** Virus replication in RPMI 1701 medium supplemented with 0, 1, 2, 5, and 10% FBS was investigated. Results given in Fig. 2 indicate that the best virus yield was obtained with the serum concentration in the medium at 1 to 2%. Low virus yields resulted when serum was omitted.

**Effect of MOI on virus growth rate.** The multiplicity of inoculum (MOI), defined as the ratio of virus TCID₅₀ to a single host cell, was calculated according to Klein et al. (12). Different MOI values were investigated for their influence on HSV growth. The highest HSV growth rate resulted with an MOI of 0.1 to 1.0 (Fig. 3).

**Culture conditions.** To obtain the maximum yield of virus, cultures were infected while in the rapid growth phase with a high cell viability (80%). Other experiments demonstrated that there was little or no difference in virus growth whether the culture supernatant fluid was completely changed for fresh medium or left unchanged prior to virus inoculation. These findings have a practical value when growing HSV in large amounts of suspended lymphoid cells. As expected, conditions that supported rapid cell metabolism also sufficed for rapid virus replication.

**Large-scale infected cultures.** In preliminary experiments, the virus was grown under controlled and uncontrolled pH conditions in fermentor units. Since the cell cultures were fed daily with fresh medium and the virus infection took only 48 hr to reach maximum levels, there was no need for exacting pH control. The pH required for cell and virus growth ranged from 6.7 to 7.0.

The conditions for optimal cell and virus growth in fermentor units were as follows. (i) Infected cultures (i.e., cells and supernatant fluid) were used to seed the fermentor units (Fig. 1). (ii) MOI of 0.1 was used for inoculation; to attain this ratio of virus to cells, the cell cultures often had to be diluted with fresh RPMI 1701 medium plus 1% FBS. (iii) Results given in Fig. 4 indicated that in noninfected cultures there was an increase in total and viable cells; in the infected cultures, cells did not multiply. There was a decrease in the viable cell concentration and an increase in the dead cell concentration as the virus infection progressed. The total cells in the culture decreased slightly and an initial cell viability of 90% was reduced to 20% by 48 hr after the virus infection.

The virus concentration was determined by three methods (Fig. 5). Quantitative measurements of the infective HSV particles, calculated as TCID₅₀ or PFU, showed that there was an increase of 2 log₁₀ of infective virus units after 48 hr. A progressive increase in infective virus in the supernatant fluid and within the cells was observed after the 9th hr and it reached its peak after 48 hr. The quantity of virus measured as PFU per milliliter was always higher but parallel to the measurements by TCID₅₀ per milliliter. The relative increase of the infective virus was greater in the supernatant fluid than in the cells.

Virus particles could not be detected by electron microscopy with negative staining until 6 hr after the infection began. The number of VLP per milliliter was higher in the supernatant fluid than in the cells. The VLP count was higher by 3 to 4 log₁₀ than that attained by calculating either the TCID₅₀ or the PFU.

The percentage of disintegrating cells detected by electron microscopy increased during the course of the infection and paralleled but was slightly higher than the percentage of dead cells calculated by the trypan blue exclusion method. There were increases both in the total number of cells and dead cells containing HSV (Fig. 6).

**Polymerase studies.** The DdP activity increased in concert with progression of the HSV infection (Table 1). The increase in DdP activity was found in both culture supernatant fluid and cell lysate but was higher in the supernatant fluid. In control experiments, the activity of DdP was low in noninfected cells with either high (90%) or low (20%) cell viability. The DdP activity in cultures with high viability remained almost constant for at least 48 hr, but the enzyme activity decreased as the percentage of dead cells in the culture increased. Very low RdP activity was found in HSV-infected cells.

**Electron microscopy.** Sections were made of cells taken at the same time intervals and were examined by electron microscopy. No intracellular changes were detected until 6 hr after infection when the first intranuclear particles were observed. At this time, about 7% of
5 ml FROZEN HSV CULTURE (AT -70 C)

STEP 1: HELO MONOLAYER

VIRUS SEED OR STOCK PRODUCTION
16 oz. PRESCRIPTION BOTTLE, 30 ml CULTURE VOLUME
CELL GROWTH PERIOD: 1-3 DAYS
HSV GROWTH PERIOD: 2 DAYS

5 ml FRESH HSV CULTURE

STEP 2: RPMI CELL LINE 8226

VIRUS SEED, STOCK PRODUCTION OR LABORATORY SCALE EXPERIMENTS
500 ml SPINNER FLASK; 150 ml SUSPENDED CULTURE
CELL GROWTH PERIOD: 4-6 DAYS, CULTURE IN THE GROWTH PHASE.
HSV GROWTH PERIOD: 2 DAYS

FRESH HSV CULTURE

STEP 3: RPMI CELL LINE 8226

VIRUS LARGE SCALE PRODUCTION
14 LITERS MICRO FERM FERMENTOR, 6 LITERS SUSPENDED CULTURE
CELL GROWTH PERIOD: 4-6 DAYS, CULTURE IN THE GROWTH PHASE.
HSV GROWTH PERIOD: 2 DAYS

STORAGE OF HSV CULTURE AT -70 C IN 5 ml PORTIONS
FROZEN HSV CULTURE

FIG. 1. Steps in propagation HSV. Step 2 or 3 were infected from fresh or frozen HSV culture. The frozen cultures were thawed rapidly in water bath at 37 C prior to infection.

Fig. 2. Effect of different FBS concentration on HSV production in Spinner flask culture.

the cells contained VLP. Approximately 80% of the cells contained viruses 48 hr after infection (Fig. 7). Some of the cells are disintegrating. After 24 and 48 hr, crystalline patterns of virus were observed in the nuclei of some cells (Fig. 8). Most of the virus particles in the nuclei had a distinct hexagonal shape and none had an external envelope. In most mature particles a core of nucleic acid was present. Apparently the virus particles were enveloped as they passed through the outer membrane of the nucleus since all VLP in the

Fig. 3. Effect of different MOI on HSV propagation in Spinner flask culture.
FIG. 4. Cell counts in HSV-infected and noninfected RPMI cell line 8226 bench scale culture.

FIG. 5. Kinetics of HSV production in bench scale RPMI cell line 8226 culture.

FIG. 6. Per cent of dead cells, disintegrating cells, and cells containing virus in bench scale RPMI cell line 8226 culture.

Table 1. Deoxyribonucleic acid (DNA)-dependent DNA polymerase (DdP) activity in herpes simplex virus (HSV)-infected and noninfected RPMI cell line 8226 in bench scale culture.

| Time (hr) after infection | Control: noninfected cultured HSV-infected culture cells a | "H-thymidine triphosphate counts per min per μg of protein |
|--------------------------|----------------------------------------------------------|----------------------------------------------------------|
|                          | Control noninfected culture                               | Dead culture                                             |
|                          | Viable cells                                             | In supernatant fluid                                     |
|                          | Dead cells                                               | In cell lysate                                           |
| 0                        |                                                          |                                                         |
| 3                        | 380                                                     | 300                                                     |
| 6                        | 300 (10)c                                               | 300                                                     |
| 9                        | 300                                                     | 1,000                                                   |
| 12                       | 250                                                     | 1,000                                                   |
| 24                       | 70 (80)                                                  | 1,650                                                   |
| 30                       | 312                                                     | 2,250                                                   |
| 48                       | 342                                                     | 3,600                                                   |
|                          |                                                         | 300                                                     |
|                          |                                                         | 100                                                     |
|                          |                                                         | 100                                                     |
|                          |                                                         | 150                                                     |
|                          |                                                         | 340                                                     |
|                          |                                                         | 1,400                                                   |
|                          |                                                         | 2,100                                                   |
|                          |                                                         | 2,700                                                   |

a No DdP activity was found in the supernatant fluid.

b This column does not correlate with hours after infection column.

c Numbers in brackets represent per cent of dead cells in culture.

cytoplasm and attached to the cell membrane had envelopes (Fig. 9).

DISCUSSION

Scaling up of cell cultures from laboratory scale to bench and pilot plant scales is usually beset with questions concerning maintenance of sterility, mixing, aeration, best inoculation levels, maintenance of optimal growth rates and viability, separation of products, and
FIG. 7. An ultrathin section made after 48 hr of infection with HSV. More than 80% of the cells were disintegrating and contained virus particles. Abbreviations: DC, disintegrating cell with broken cytoplasmic membrane; NDC, nondisintegrating cell with intact membrane; NV, nuclear, naked virus; EV, extracellular enveloped virus. × 9,000.

others (1, 4). Unexpectedly there were only minor problems in scaling up the growth of HSV in contrast to the frustrations associated with culturing the related herpes virus—EBV. The cells and the HSV grew in the same medium with the same type and concentration of serum. The fact that there was no need to change the culture medium prior to infection with HSV eliminated many technical problems. However, the present model system may not be adaptable for producing other viruses that can be grown in established cell lines, because some viruses require a complete replacement of the culture medium, e.g., FMDV in BHK culture (26, 39). This requirement complicates and increases the expense of prop-
Occasionally intranuclear crystalline patterns of VLP were observed in ultrathin sections of cells 48 hr after infection with HSV. The packing seems to indicate the hexagonal shape expected for icosahedral virions. Note that not all of the VLP contain electron-dense cores; however, these viruses may have been cut tangentially. NM, nuclear membrane; CM, cytoplasmic membrane. × 39,000.
Fig. 9. An ultrathin section of a cell 24 hr after infection showing intranuclear naked viruses and extranuclear and extracellular enveloped particles. NV, nuclear naked virus; EV, extracellular enveloped virus. × 24,000.

Agitation of viruses in large suspension culture units.

Nahmias et al. (24) have reported that HSV can replicate in fresh human leukocytes in vitro provided the cells are stimulated with phytohemagglutinin. We have found that HSV can replicate in permanent human lymphoid cell lines without stimulation. The HSV yields obtained by this method were 10 to 15 PFU per cell. Roizman and Spear (33) have ob-
tained a higher yield of HSV in roller-bottle cultures with HEP-2 cells. They were able to produce HSV in a range of 100 to 200 PFU per cell. In this study, the relatively low virus yield may be due to the low level of MOI used. Alternatively, the RPMI 8226 cells may have the EBV genome in them since Zur Hausen and Schulte-Holthausen (40, 41) have recently found that the EBV genome was present in the "EBV-negative" Burkitt lymphoma cell line, Raji, and in fresh biopsy materials from Burkitt lymphoma as well as from anaplastic carcinoma of the naso-pharyngeal region. The presence of the EBV genome may perhaps interfere with HSV replication in analogy to the limited replication of EBV in many lymphoid cell lines. Recently Rabson et al. (27) reported on a growth inhibitor of Herpesvirus hominis found in extracts of the ALI cell line, an EBV-positive Burkitt lymphoma cell line. Such an inhibitor may be produced by other lymphoid cell lines with an apparent EBV infection, thus preventing replication of HSV to different extents depending upon the degree of infection. Further experiments may determine whether the relatively low HSV yield can be improved or if this is inherent for these lymphoid cells.

The calculation of the ratio VLP/ PFU indicated that 12 hr after the infection this value was higher than expected: 10^3 to 10^4 VLP/PFU. Thirty and 48 hr after the virus infection, 10^3 to 10^4 VLP/PFU were calculated. Perhaps at the beginning more noninfective virions were present in the culture; thus, the ratio of VLP/ PFU was high. As the infection progressed, complete virus particles were released resulting in a much lower VLP/ PFU ratio.

All the methods used for characterization of the HSV infection (different virus counts, cell counts, and electron microscopic observations of cell sections) indicated that there was a progressive HSV infection, although it was not synchronous.

Preliminary screening for a suitable host cell line for HSV among the permanent human lymphoid cell lines in the RPMI cell bank indicated that RPMI cell line 8226 was one of the best hosts. Indirect biological tests have shown that this is an atypical cell line (9, 16, 22). Characteristics of RPMI cell line 8226 that may explain its ability to support the growth of HSV are a lack of immunoglobulin production (it produced only gamma-type light chains), possible inability to make interferon, and undetectable levels of infection with EBV; EBV may cause inhibitory cross-reactions with HSV.

Although it was previously reported (22) that RPMI cell line 8226 cells grew for long periods in serum-free medium, the available cultures in a large-scale unit failed to grow in a medium without serum. Nevertheless, the serum concentration needed for growth was low: 2% compared with 5% required for reasonable growth rates of other human lymphoid cell lines. The presence of HEPES buffer in RPMI 1701 medium prevented rapid pH changes associated with bicarbonate buffering of the standard RPMI 1640 medium (20).

The low RdP activity found in HSV-infected cultures might be expected when dealing with a DNA virus (11). The increased DdP activity in HSV-infected cultures needs further investigation to determine whether it was due to virus induction of a host or a virus enzyme.

A good yield of HSV and elevated enzyme activity in large-scale lymphoid cell culture systems could provide sufficient materials for further biochemical and immunological analyses.

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