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Ultracentrifugal Inoculation of Herpes Simplex Virus

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By ultracentrifugation of 30 ml of highly dilute suspensions of herpes simplex virus (HSV) directly onto monolayer cultures grown in centrifuge tubes, infectivity was significantly greater than without centrifugation. Ultracentrifugation at 20,000 to 25,000 rpm (28,000 to 45,000 × *g*) for 1.5 to 2.3 h was utilized with good preservation of cultures. With low-speed centrifugation at 3,000 rpm (1,100 × *g*), infectivity was almost 10-fold greater than without centrifugation. With ultracentrifugal inoculation, infectivity was about 100-fold greater than without centrifugation. Ultracentrifugal inoculation permitted the detection of HSV at concentrations as low as 0.05 plaque-forming units per ml. Similarly, ultracentrifugal inoculation of cultures was almost 100-fold more sensitive a method of detecting infectious HSV than was pelleting HSV from dilute suspensions followed by resuspension and inoculation of cultures. Ultracentrifugal inoculation of cultures may permit the isolation of HSV in situations where virus cannot be detected by ordinary means and may prove applicable to the study of other viruses.

The detection of small amounts of viruses is of importance in research and in clinical medicine. Currently, radioimmunoassay and in situ nucleic acid hybridization or renaturation have been employed as sensitive techniques to detect the presence of viral antigens or nucleic acids (4, 7, 13). Immunoelectron microscopy has been employed to detect virus particles (3). Although with isolation techniques it is theoretically possible to detect a single infectious virus particle, the sensitivity of virus isolation testing as generally performed is limited.

We have recently utilized a technique, however, which greatly increased the sensitivity of tissue culture isolation of herpes simplex virus (HSV). To evaluate the isolation of infectious HSV from extremely dilute virus suspensions, the effect of ultracentrifugation of virus onto susceptible monolayer cells was investigated. Results were quantitatively compared with those obtained with standard isolation testing.

MATERIALS AND METHODS

Virus. In initial experiments HSV type 1 (HSV-1; KOS strain) was studied. Stock virus was grown in primary rabbit kidney (RK) cell monolayer cultures and was harvested by freeze-thawing the cultures. Supernate virus was clarified by low-speed centrifugation (3,000 rpm), divided into aliquots, and frozen at −70°C. The infectivity titer of the stock HSV was 5 × 10^6 to 8 × 10^6 plaque-forming units (PFU) per 0.1 ml, as determined in RK cells. The Patton strain of HSV-1 and the MS strain of HSV type 2 (HSV-2) were studied after similar preparation. Titers of these viruses were 10^7 PFU/0.1 ml and 10^5 PFU/0.1 ml in RK cells, respectively.

Growth of cells. For centrifugation studies, monolayer cultures were grown in sterile Oakridge style polycarbonate centrifuge tubes (25.3 by 89.6 mm, Sorvall, Newton, Ct.). Vero cells (Flow Laboratories, Rockville, Md.) in growth medium (medium 199 with sodium bicarbonate and antibiotics added and supplemented with 10% fetal calf serum) were seeded into the sterile tubes. To each centrifuge tube, 2 to 3 ml of cell suspension (10^5 cells per ml) was added. Similarly, 2 to 3 ml of primary RK cells (5 × 10^5 cells per ml) prepared by standard procedures (12) and suspended in Eagle minimal essential medium supplemented with sodium bicarbonate, antibiotics, and 10% calf serum was inoculated into other tubes. Tubes were incubated at about a 45° angle. RK cells were also cultured in plastic petri culture dishes (60 by 15 mm). Culture dishes were incubated in a humidified atmosphere of 5% CO_2, and all cultures were incubated at 37°C. When monolayers were complete, growth medium was replaced with maintenance medium (medium 199 supplemented as described above but with 5% fetal calf serum for Vero cells or Eagle basal medium supplemented as described above but with 5% calf serum for RK cells).

Infectivity determinations. After adequate growth of monolayer cultures, the infectivity of serial 10-fold dilutions of stock HSV was determined by ascertaining (i) the number of PFU in petri-dish cultures; (ii) the 50% tissue culture infective dose ($TCID_{50}$) in centrifuge tube cultures inoculated with 0.1 ml of virus suspension and not centrifuged; (iii) the $TCID_{50}$ in centrifuge tube cultures inoculated with 30 ml of virus suspension and not centrifuged; (iv) the $TCID_{50}$ in centrifuge tube cultures inoculated with 30 ml of virus suspension and low-speed centrifugation;

and (v) the $TCID_{50}$ in centrifuge tube cultures inoculated with 30 ml of virus suspension and ultracentrifugation (Fig. 1). Determination of the number of PFU in 10-fold dilutions was performed in duplicate. For each type of tube culture titration, four tubes were inoculated for each dilution of virus. Titration by these procedures was performed multiple times in Vero and in RK cells with fresh virus used each time.

For PFU determination in petri-dish cultures, standard procedures were employed (8). In brief, the medium was decanted, and 0.1 ml of virus suspension was added. Adsorption at 37°C in a CO_2 incubator was carried out for 1 h, after which 5 ml of 0.5% methylcellulose overlay was added. After incubation in a CO_2 incubator for 4 days, the methylcellulose was removed and 5 ml of 0.1% neutral red in tris(hydroxymethyl)aminomethane buffer was added. Plaques were counted after an additional 4 h of incubation.

Inoculation of centrifuge tube cultures. Titrations in tube cultures were performed by inoculation of 0.1 or 30 ml of virus suspension after decanting the medium from the tubes. Adsorption was at room temperature for 1.5 to 2.3 h with the tubes at a 45° angle, after which virus suspensions were decanted and fresh medium was added. Titrations of stock virus in petri-dish cultures provided estimates of the number of PFU inoculated into each tube.

Low-speed centrifugation. Centrifuge tube cultures for low-speed centrifugation were inoculated with 30 ml of virus suspension and were placed in a Sorvall SS-34 rotor with the monolayers positioned to receive the centrifugal force. Tube cultures were centrifuged at 3,000 rpm (1,100 × *g*) for 10 min at 20°C. After centrifugation, tubes were positioned as described above for the uncentrifuged tubes and kept at room temperature for a total inoculation time of 1.5 to 2.3 h.

Ultracentrifugation. Centrifuge tube cultures inoculated with 30 ml of virus suspension were positioned in a fixed-angle Beckman 60 Ti rotor so that monolayers received the maximal centrifugal force. That is, monolayer cultures were positioned so that suspended material would be pelleted onto the monolayers. Ultracentrifugation (Sorvall, OTD-2) at 20,000 to 25,000 rpm (28,000 to 45,000 × *g*) was for 1.5 to 2.3 h at 20°C. After ultracentrifugation the virus suspensions from these tubes and from uncentrifuged and low-speed centrifuged tubes were decanted, and 2 to 3 ml of the appropriate maintenance medium was added.

All tubes were incubated at 37°C and examined daily for the development of cytopathic effect typical of HSV. Virus infectivity titers were determined by the method of Reed and Muench (9).

RESULTS

Effect of volume inoculated. Since in the evaluation of ultracentrifugal inoculation (UCIN) of very dilute virus suspensions it was necessary to inoculate tubes with large volumes (discussed below), initially the effect of volume of suspension inoculated on infectivity was evaluated. Concentration of virus was kept constant, and tube cultures were not centrifuged. The mean infectivity titer determined in centrifuge tube cultures which were inoculated with 30 ml was 7.2 log $TCID_{50}$, 0.9 log unit greater than that of 6.3 $TCID_{50}$ after inoculation of similar tube cultures with 0.1 ml. In the following centrifugation studies, all tube cultures were inoculated with 30-ml samples.

Effect of low-speed centrifugation. In tubes which had been centrifuged at 3,000 rpm (1,100 × *g*), the log $TCID_{50}$ titer of stock virus was almost 10-fold greater than in similar tubes which had not been centrifuged (Table 1). Infectivity titers after centrifugation at 1,000 rpm (100 × *g*) were slightly below and after centrifugation at 5,000 rpm (3,000 × *g*) slightly above those after centrifugation at 3,000 rpm (data not shown).

To determine whether centrifugation of mon-

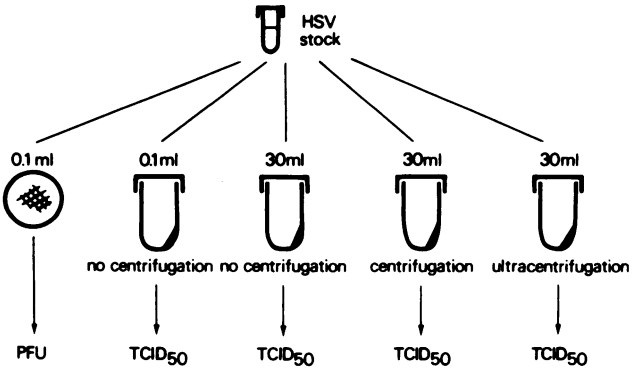

FIG. 1. *Experimental design of this investigation. Infectivity titer of stock HSV was determined by several methods, including UCIN of monolayer cultures. The determination of the number of PFU in culture dishes at the time of each series of centrifuge tube culture inoculations permitted estimates to be made of the concentration of PFU in centrifuge tube cultures.*

TABLE 1. *Effect of centrifugation and ultracentrifugation of HSV suspensions on infectivity titer*

Virus strain tested	log $TCID_{50}$/30 ml, mean ± SE[a]		
	No centrifugation	Low-speed centrifugation[b]	Ultracentrifugation[c]
KOS (HSV-1)	7.1 ± 0.2	7.9 ± 0.1	8.9 ± 0.1
Patton (HSV-1)	7.2 ± 0.3	8.1 ± 0.3	9.0 ± 0.2
MS (HSV-2)	5.2 ± 0.2	6.1 ± 0.3	7.2 ± 0.1

[a] Data obtained with RK and Vero cells were pooled. All cultures were inoculated with 30 ml of dilute virus suspension. SE, Standard error.

[b] Centrifuged at 3,000 rpm (1,100 × *g*).

[c] Centrifuged at 20,000 to 25,000 rpm (28,000 to 45,000 × *g*).

olayer cells before inoculation of virus suspension resulted in an increase of infectivity titer, monolayer cultures were centrifuged at 3,000 to 5,000 rpm before inoculation with 30 ml of virus suspension. Infectivity titers in these pre-inoculation centrifuged tubes were similar to tubes which had not been centrifuged.

Effect of ultracentrifugation. With ultracentrifugation, infectivity titers were 10-fold greater than those with low-speed centrifugation and were almost 100-fold greater than those without centrifugation (Table 1). Results from using UCIN were similar with RK and Vero cells. With RK-13 cells, however, a lesser degree of ultracentrifugal enhancement of infectivity was observed (data not shown). Ultracentrifugation of RK and Vero cell monolayer cultures before inoculation with 30 ml of virus suspension did not result in any enhancement of infectivity when compared to noncentrifuged tube cultures.

Since determination of the number of PFU/0.1 ml of diluted stock virus was made for each titration, an estimate of the number of PFU inoculated into each centrifuge tube could be made. This permitted determination of the efficacy of each method of tube titration to detect low concentrations of PFU (Fig. 2). With UCIN, a mean of 0.02 PFU/0.1 ml was detected, and on some tests as little as 0.005 PFU/0.1 ml was detected. The sensitivity of the UCIN procedure to detect infectious HSV was 100-fold greater than that of similar tubes which had not been centrifuged ($P < 0.005$, Student's *t* test).

To compare the UCIN procedure with concentration of HSV by conventional pelleting, the following procedure was utilized. Dilutions of stock HSV were made, and 30-ml samples were ultracentrifuged in sterile tubes which did not contain monolayer cultures. For each dilution four tubes were centrifuged at 25,000 rpm (45,000 × *g*) for 1.5 h at 4°C. Each tube was then decanted, and the pellet was scraped and resuspended in 0.1 ml of Hanks balanced salt solution. Centrifuge tubes with Vero cell monolayer cultures were decanted, and each resuspended pellet was inoculated into one tube. Suspensions were adsorbed to the monolayers for 1.5 h at room temperature before incubation at 37°C. Infectivity was almost 100-fold less than that obtained with the UCIN procedure (Fig. 2).

As an integral part of developing the technique of UCIN the effect of ultracentrifugation on living monolayer cultures of Vero and RK cells was evaluated. With ultracentrifugation of Vero cells there was more disruption of monolayers at speeds greater than 20,000 rpm (28,000 × *g*) than was seen with RK cells. Therefore, for

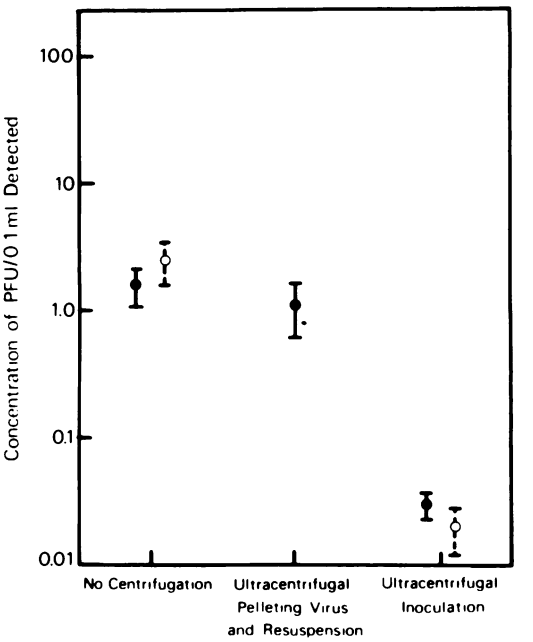

FIG. 2. *Effect of UCIN on sensitivity of detection of HSV. Logarithmic plot of minimal detection of the number of PFU for each type of procedure (mean and standard error). Centrifuge tube cultures were inoculated with 30-ml samples of dilute HSV or with the HSV pellet derived from 30 ml. Estimates of the number of PFU in each tube culture were based on the number of PFU detected in simultaneously inoculated plate cultures. A single positive tube culture was considered to indicate the detection of the appropriate concentration of PFU. By this method of analysis the sensitivity of detection between titrations in which one tube was positive or in which two tubes were positive was not differentiated. Sensitivity of HSV detection was calculated in terms of concentration of PFU rather than the total number of PFU present in the centrifuge tube cultures. Symbols: ●, Vero cells; ○, RK cells.*

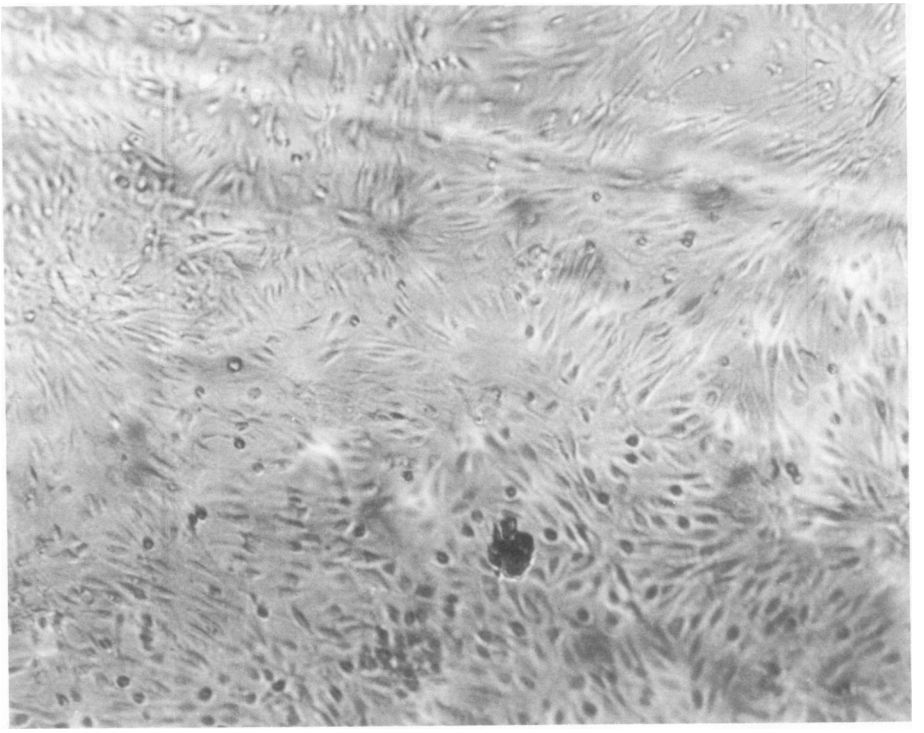

FIG. 3. *Living RK monolayer cell culture after ultracentrifugation at 25,000 rpm (45,000 × g) for 1.5 h at 20°C. ×110.*

most experiments Vero cells were ultracentrifuged at 20,000 rpm (28,000 × *g*) for 2.3 h, whereas RK cells were ultracentrifuged at 25,000 rpm (45,000 × *g*) for 1.5 h. RK cells after such an ultracentrifugation procedure are seen in Fig. 3.

DISCUSSION

In this study we present evidence that UCIN of HSV onto monolayer cultures may be employed to isolate virus from suspensions containing extremely low concentrations of infectious HSV. Ultracentrifugation did not significantly disrupt cultures and resulted in a 100-fold increase in virus detected as compared to no centrifugation. Use of ultracentrifugation permitted the isolation of HSV from suspensions which yielded consistently negative results without centrifugation. This procedure was considerably more efficient than pelleting and resuspension of virus and may prove valuable in situations where isolation of viruses, particularly from large volumes, is difficult.

Inoculation of centrifuge tube cultures with large volumes (30 ml) was utilized to permit study of HSV isolation from dilutions greater than those from which virus could be routinely isolated. In these large volumes, despite the low concentration of virus per milliliter (for example, 0.1 PFU/ml), some infectious virus would be expected to be present. This would not be the case if only 0.1 ml were inoculated, and in testing the efficacy of any isolation procedure it is obviously necessary that virus be present or all results will be negative. For example, if a virus suspension that was diluted to 10 PFU/ml was further diluted 100-fold, it would be estimated that there would be 0.1 PFU/ml. Of tubes or plates inoculated with 0.1 ml of this suspension, only 1 in 100 would be expected to be positive under the best of circumstances. Therefore, to ensure the presence of some virus in the very highly dilute virus suspensions studied, large volumes were used for the inoculation of cultures.

By determining the number of PFU in stock virus diluted to 10^{-6}, it was possible to estimate the concentrations of PFU in dilutions to 10^{-9}. This permitted estimates to be made of the level or sensitivity of detection of HSV for each isolation procedure. Sensitivity of virus isolation procedures may be expressed in terms of their being able to detect infectious virus when virus is present in a low concentration. Although fractions of PFU cannot be considered detectable,

the calculation of sensitivity of detection in terms of PFU/0.1 ml does express the sensitivity of each method.

The use of low-speed centrifugation to inoculate monolayer cultures has been studied previously (2, 5, 6, 10). Recent work on this subject, primarily with mouse cytomegalovirus, was reported by Osborn and Walker (5) and by Hudson et al. (2). Both groups of authors discussed centrifugal enhancement insofar as it applied to mouse cytomegalovirus rather than as a more general phenomenon. In fact, they used HSV as a negative control virus for which only very limited centrifugal enhancement was apparent. These authors indicated a less than eightfold enhancement of HSV infectivity in mouse embryo cells with low-speed centrifugation (2, 5). The centrifugal enhancement we observed with low-speed centrifugation was of similar magnitude. Use of ultracentrifugation of virus suspensions onto monolayer cultures was briefly discussed by Gey et al. (1), but quantitative comparisons with noncentrifuged cultures were not made.

Ultracentrifugation at 25,000 rpm (45,000 × *g*) for 1.5 h may be used to pellet HSV virions. It is probable, therefore, that the centrifugal forces utilized brought sparsely distributed virus particles in the very dilute suspensions tested into contact with the test monolayers. Similarly, with low-speed centrifugation, it is hypothesized that virus aggregates which are known to be present in HSV suspensions (11) were brought into contact with the monolayers. One can only speculate at present on the possibility that ultracentrifugation may have permitted the isolation of HSV virions that would otherwise have been noninfectious.

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