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Microtissue Culture Plaque Assay for *Herpesvirus saimiri*

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A microtissue culture method for the plaque assay of *Herpesvirus saimiri* has been developed. Virus titrations carried out in Microtest II tissue culture plates (Falcon) yielded reproducible results that agreed well with those obtained by employing macrocultures. The described method is quantitative, reproducible, economical, and suitable for routine assay of large numbers of virus samples.

Herpesvirus saimiri (HVS), an agent isolated from cell cultures of squirrel monkey (*Saimiri sciureus*) kidney (6, 8), induces rapid malignant lymphosarcoma or acute lymphocytic leukemia, or both, in marmosets (*Saquinus oedipus*, *S. fusciollis*, *S. nigricollis*) and owl monkeys (*Aotus trivirogotus*) (2, 5, 6, 8-11). Evidence, fulfilling Koch's postulates, has been obtained (7), indicating that HVS is the etiological agent of the lethal disease(s) in these nonhuman primates. Therefore, the possibility exists that HVS may provide an important primate model for the study of herpesvirus in human neoplasia. To faciliate further studies on HVS, there is a need for a sensitive, reproducible, quantitative, and economical tissue culture assay for this oncogenic deoxyribonucleic acid virus.

HVS replicates in both simian (6, 8) and human cell cultures (4) with the production of a typical cytopathic effect. The changes are characterized initially by the formation of discrete clusters of large, rounded, and refractile cells which subsequently lyse and develop into plaques, each having a ring of rounded cells at the periphery.

Tissue culture assays for HVS are usually carried out on indicator cells seeded in test tubes (4, 6) or in 60-mm petri dishes (3). The former procedure is convenient but semiquantitative. The latter is quantitative but unsuitable for routine handling of large number of virus samples. In the present report we describe an accurate, reproducible, and quantitative microtissue culture method for the titration of HVS.

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MATERIALS AND METHODS

Cell cultures. Primary owl monkey kidney (OMK) cells were obtained from Bionetics Research Laboratory (Kensington, Md.). They were grown in 75-cm^2 Falcon plastic flasks, in RPMI 1640 medium, containing 15% fetal calf serum, 10 U of mycostatin per ml and 100 μg each of penicillin, streptomycin, and neomycin per ml. The cultures were incubated at 37 C in a humidified, 5% CO_2 atmosphere. The cells were subcultured weekly, and for the present study, cells from the 3rd to 12th passage were used.

Plastic Microtest II tissue culture plates (Falcon), with 96 circular flat-bottom wells (7 mm in diameter), were used for the preparation of microcultures. Plastic petri dishes (Falcon, 35 mm in diameter) were used for macrocultures. Freshly trypsinized OMK cells were seeded at a density of 2×10^4 cells (in 0.2 ml) per microculture and 2×10^5 cells (in 2.0 ml) per macroculture. All cultures were incubated at 37 C in a humidified, 5% CO_2 atmosphere for 3 to 4 days. At this time, the cells approached confluency and were used for infectivity studies.

Virus stocks. A stock of HVS was obtained from D. V. Ablashi, National Cancer Institute, and was propagated in OMK cells grown in 75-cm^2 Falcon plastic flasks. The flask cultures were each infected, when confluent, with 1.0 ml of undiluted virus. After adsorption for 30 min at 37 C on a rocker platform, 15 ml of medium was added to the flasks and they were incubated at 37 C in a humidified, 5% CO_2 atmosphere. A complete change of medium was made on day 2 postinfection. On day 7 to 9, when 80 to 90% of the cell sheet was destroyed, the remaining cells were scraped into the medium and pelleted by centrifugation at $350 \times g$ for 10 min. The supernatant fluid was withdrawn, with the exception of 2 ml, and placed in an ice bath. The cell pellet was resuspended in the remaining 2 ml of medium and frozen-and-thawed twice. Cell debris was removed by centrifuging at $350 \times g$ for 10 min. The supernatant fluids were combined and stored at -70 C until use.

Plaque assay. Virus titrations were carried out in both micro- and macrocultures of OMK cells. Serial 10-fold dilutions of the virus were made in cell culture medium without mycostatin. Prior to infection, the medium was aspirated. Each virus dilution was inoculated into triplicate micro- and macrocultures, in 0.05 ml and 0.10 ml, respectively. After adsorption for 30 min, the infected micro- and macrocultures were overlaid with 0.2 ml and 2.0 ml, respectively, of 1.5% methylcellulose (Fisher, 4000 centipoise) in cell culture medium, further supplemented with 2% tryptose phosphate broth (Gibco). For the experiment described in Fig. 3, 0.5% agar was used in addition to methylcellulose. All infected cultures were incubated in a 5% CO_2, humidified atmosphere at 37 C. Plaques were counted either microscopically at day 7 to 9 postinfection, under a lower-power inverted light microscope, or macroscopically at day 12 to 14 after removal of the overlay and a 10-min staining with 0.5% crystal violet in 70% methanol. The plaques, at the later stage, have grown to 0.5 to 1.0 mm in diameter, and were easily countable with the naked eye.

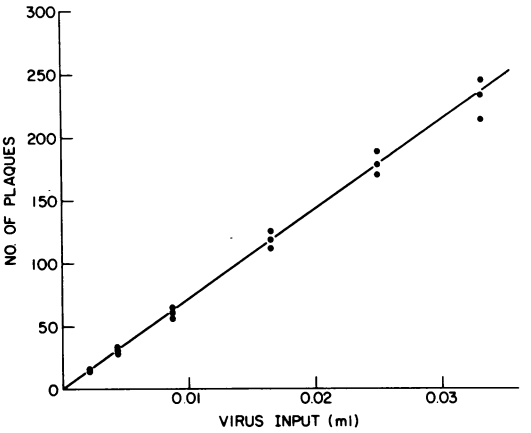

FIG. 1. *Relationship between plaque count and virus infective dose. OMK monolayer cultures in 35- by 10-mm petri dishes (Falcon) were infected in triplicate with varying amounts of a virus stock in a constant 0.1-ml inoculum.*

RESULTS

Prior to investigating the assay of HVS in microtiter plates, the relationship between virus input and plaque formation was determined. OMK monolayers in 35- by 10-mm petri dishes were infected with 0.1 ml of various dilutions of virus. Counts on triplicate cultures revealed that there was a completely linear relationship between the dose of virus and the number of plaques formed even at end-point dilutions (Fig. 1).

Infection of OMK cells in microtiter plates with HVS resulted in the appearance of plaques similar to those previously described (7). Morphological changes in the cells were evident on day 3 to 4 after infection, and a typical plaque is shown in Fig. 2. Plaques could be counted microscopically by day 7 to 9 postinfection and macroscopically at day 12 to 14.

Since the plaques were easily observed in microtiter plates, studies were undertaken (i) to determine the reproducibility of the microculture assay, and (ii) to compare this microassay with the plaque assay in petri dishes. Three different pools of virus were titrated in a series of parallel tests by using OMK cells prepared from different kidneys. Counts were made on triplicate wells at end-point dilutions of the virus, since the maximum number of countable plaques per well was 20. The results of these tests are summarized in Table 1 and show that there were no significant variations in titer with samples from any one pool of virus when assayed in the microtiter plates. In addition, the titers in these assays were comparable to those in the parallel assays in macrocultures.

The effects of different overlays on the microculture assay system were also investigated. It can be seen (Fig. 3) that under a methylcellulose overlay the number of plaques increased sharply between day 4 to 6, reached a peak by day 7, and then remained stable. Without an overlay, the number of plaques were initially somewhat lower but by day 9 became too numerous to count. This continuous rise resulted from the appearance of new secondary plaques which were smaller in size than those initially seen. In contrast, the agar overlay inhibited the development of plaques. The number remained at an initial low level, and by the 10th day postinfection, there were 20-fold fewer plaques with agar than with methylcellulose. Methylcellulose was, therefore, used for all subsequent assays.

Additional factors that might have an effect on the assay system were also investigated. The results revealed that the number of days in culture (4–8 days), the passage number (passage 3–11), and the initial plating density (10^4 to 4×10^4) of the OMK cells did not affect the titer. In addition, variations in serum concentration, from 5 to 15%, in either McCoy 5A or RPMI 1640 medium, did not produce any significant differences in the titer of a given virus sample. The length of time necessary for the complete adsorption of the virus to the cells was 10 min (Fig. 4), and additional adsorption time of up to 2 h did not result in an increase in titer.

DISCUSSION

The results presented in this study show that HVS can be quantitatively and reproducibly

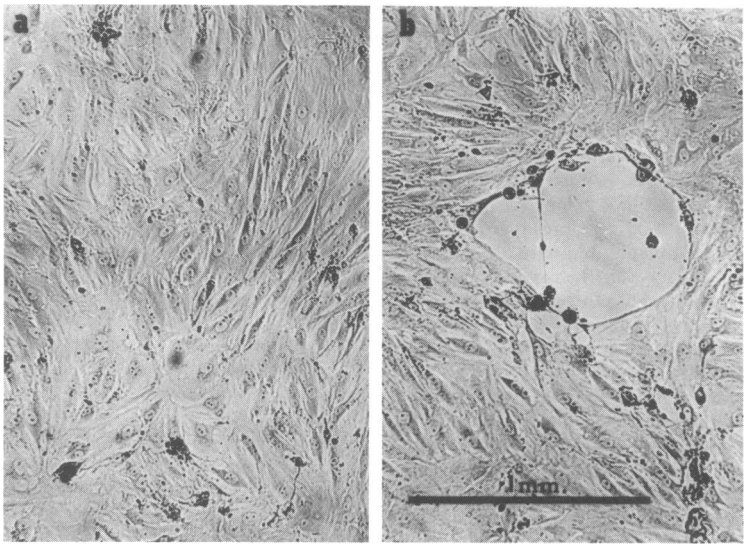

FIG. 2. *Light micrograph of (a) a normal monolayer of OMK cells in microculture and (b) a plaque in a matched culture but infected with HVS at day 5 postinfection.*

TABLE 1. *Parallel virus titrations in macro- and microcultures*

Virus stocks	Plaque forming units per ml in:				
	Macrocultures		Microcultures		
	Test 1	Test 2	Test 1	Test 2	Test 3
V	1.1×10^5	1.2×10^5	1.2×10^5	1.1×10^5	0.9×10^5
VIII	2.0×10^5	1.9×10^5	1.9×10^5	1.9×10^5	1.2×10^5
IX	1.8×10^4	1.7×10^4	1.6×10^4	1.7×10^4	1.8×10^4

titered in microtissue cultures of OMK cells. The titer of a given virus sample, as determined by this method, is based on the average of triplicate plaque counts, made at end-point dilutions of the virus. This method of quantitation was valid since there was a linear relationship between the infective dose and the number of plaques observed. Furthermore, parallel titrations carried out in macrocultures and employing calculations from plaque counts at several virus dilutions gave essentially the same titers.

The effects of different overlays on the microtissue culture assay system were also compared. The results indicated that, in the absence of an overlay, plaques normally appeared in an infected culture 3 to 4 days postinfection. The developing plaques could be unmistakably identified and counted microscopically beginning on the 6th to 7th day postinfection. However, by this time secondary plaques began to appear, making accurate quantitation difficult. The use of an agar overlay was not suitable, since agar was strongly inhibitory. When a methylcellulose overlay was used, however, plaques appeared earlier, and a higher and constant titer was achieved. Thus, the incorporation of a methycellulose overlay into the assay system was both necessary and advantageous.

Since stable titers were obtained under a methylcellulose overlay, quantitation of plaques could be reliably made either microscopically at day 7 or macroscopically on day 12 postinfection. Both methods of quantitation yielded identical titers. The former procedure was preferred, since triplicate wells per virus sample could be rapidly scored under the microscope.

The adsorption of HVS to OMK cells was found to be complete within 10 min, under the conditions described. Further adsorption did not result in a higher titer. Thus, the usual adsorption time of 1 to 2 h, as carried out in

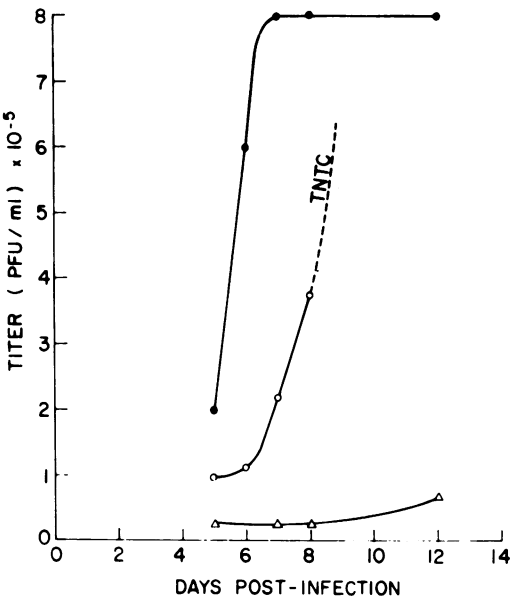

FIG. 3. *Effect of overlays on the microtissue culture assay system. A virus pool was titered in the presence of a methylcellulose (●), fluid (○), or agar (△) overlay. Each assay was carried out in quadruplicate. The titers on various days postinfection are shown.*

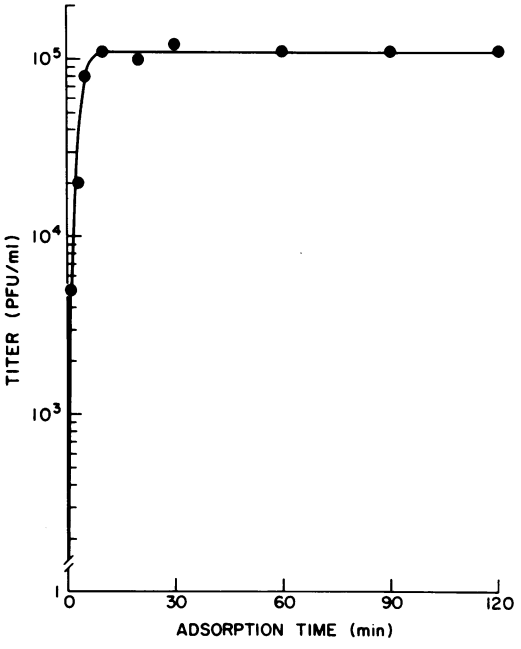

FIG. 4. *Effects of adsorption time on virus titer. A pool of virus was titered under standard conditions in microtissue cultures, except the virus adsorption period was varied from 1 to 120 min. The average titers obtained from quadruplicate determinations are shown.*

previous studies (3, 4, 6), could be considerably reduced.

Other factors that might affect virus titer in the microtissue culture assay system were examined. Small variations in the age of the OMK cells and the concentration of serum in the overlay medium used were found to produce no appreciable effects on the titer of a given virus stock. The OMK cells used were from early passages of primary cultures, since the viability declined after the 12th passage. Other susceptible and stable monkey kidney cells, such as African green and squirrel monkey kidney, which are known to respond to infection by HVS with similar cytopathogenic effect (3, 4), could conceivably be employed in place of the OMK cells used in this study.

The present microtissue culture assay considerably conserves materials, reagents, and time. As many as eight different assays can be simultaneously performed in triplicate on a single microplate of OMK cells. Furthermore, the method offers great sensitivity. Only 50 μliters of virus inoculum is required to infect one microculture. Thus, the assay is capable of detecting small quantities of low-titered virus and is applicable for use in virus infectivity, replication, inhibition, and purification studies.

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