Plaque Assay of *Herpesvirus Hominis* on Human Embryonic Fibroblasts* (33932)

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Several techniques for plaque assay of Herpesvirus hominis have been described, but none appears to offer a simple, sensitive assay which fulfills the requirements prescribed by Cooper (1). Primary cell cultures and sequential agar overlays were more successful than other cell cultures and single overlays (2, 3). However, the preparation of primary cell cultures and the use of multiple overlays are expensive and cumbersome steps for a plaque assay. The present paper describes an assay employing human embryonic fibroblasts with an agarose overlay medium which yields clear, easily discernible plaques, and fulfilled the criteria which Cooper (1) found essential for a useful plaque assay.

Materials and Methods. Cells. The HET (human embryonic tonsil) and WI-38 fibroblasts were obtained from Flow Laboratories, Inglewood, California. FT-1 and H716 are fibroblast strains derived in our laboratory from human fetal tonsil and parotid gland, respectively. M-HeLa, HeLa-229, McCoy, and L cells were received from Dr. G. E. Kenny (4). No strains of mycoplasmata were isolated from the cells, which were cultured routinely before use.

Media. Cells were grown in Eagle's minimum essential medium with 10% fetal calf serum, 6.6 m*M* sodium bicarbonate, 100 μg/ml of streptomycin, 100 units/ml of penicillin and 10 units/ml of Mycostatin. Virus was diluted in medium containing 2% agamma globulin calf serum (MEM2) in place of fetal calf serum.

Overlay medium consisted of equal parts of (i) double strength MEM2 with 8.0 m*M* sodium bicarbonate, and (ii) 0.6% agarose (SeaKem; Bausch and Lomb, Inc., Rochester, N. Y.) in distilled water. The components were brought to temperature in a 45° water bath and mixed. The complete overlay medium was held at 45° until used.

Virus strains. Three herpesvirus strains isolated in our laboratory and four strains kindly supplied by Dr. A. J. Nahmias, Emory University School of Medicine, were used. UW-165 was isolated from an adult throat culture and UW-168 from sputum of a case of recurrent pneumonia. Fluorescent antibody studies suggest that they are Type 1 respiratory strains (5), as are two of the Emory strains. UW-268 was isolated from a congenitally infected newborn infant and appears to be a Type 2 genital strain (5), as are the other two Emory strains. UW-168 was used for most studies and the other strains for comparisons.

The Type 1 poliovirus (Mahoney strain) was obtained from Dr. G. E. Kenny, University of Washington.

Virus was propagated in HET or M-HeLa cells and stocks of supernatant fluid, filtered to remove cells, were stored at —80°. No mycoplasmata could be isolated from these stocks upon culture.

Plaque assay. Monolayers were prepared by seeding 60-mm plastic tissue culture dishes (Falcon Plastics, Los Angeles, Calif.) with 5×10^5 cells in 5 ml of growth medium/plate. After overnight incubation at 37° in 2.5% CO_2, the growth medium was removed and each plate was inoculated with 0.1 ml of suitably diluted virus. Inocula were allowed to adsorb for 30 min at room temperature; adsorption for longer periods did not increase plaque counts. After adsorption, 5 ml of overlay medium at 45° was added to each plate. When the overlays had solidified, the plates were incubated at 37° in 2.5%

* Supported by Grants HD 03367-01A1 from the National Institute of Child Health and Human Development and GSRF-171 from the University of Washington.

TABLE I. Plaque Assay of Herpesvirus Strains on Various Cells.

Virus	Cells						
	HET	FT-1	H 716	WI-38	M-HeLa	McCoy	L
UW-168	1.9×10^{7}[a]	—[b]	—	—	2.8×10^{6}	—	—
	2.4×10^{7}	—	—	—	9.6×10^{5}	—	—
	1.2×10^{7}	1.0×10^{7}	1.5×10^{7}	1.2×10^{7}	—	1×10^{6}	—
	7.3×10^{6}	—	—	—	—	—	5.4×10^{8}
UW-165	1.7×10^{8}	—	—	—	1.5×10^{6}	—	—
	2.1×10^{8}	—	—	—	—	—	5.8×10^{5}
UW-268	3.8×10^{3}	—	—	—	2.9×10^{2}	—	—

[a] Plaque-forming units (pfu/ml).
[b] Not done.

CO_2 for 72–96 hr. The monolayers were then fixed by the addition of 2 ml of 10% formalin/plate for 10 min at room temperature. The overlays were discarded, the plates were washed with tap water, stained with 1 ml of 1% crystal violet in 20% ethanol/plate (6), washed in tap water and allowed to dry. Plaques were counted with the aid of an automatic colony counter ("Markounter"; Aztec Instruments, Inc., Westport, Conn.)

For reisolation of virus, plaques were removed from plates with capillary pipettes, transferred to 1 ml of medium, and assayed by a tube dilution technique.

Neutralization. Plaque reduction. Dilutions of hyperimmune guinea pig serum (Microbiological Associates, Inc., Bethesda, Md.) and undiluted normal guinea pig serum were mixed with 10^2, 10^3 and 10^4 plaque-forming units (pfu) of virus, the mixtures were allowed to stand 1 hr at room temperature and assayed for unneutralized virus by the plaque technique.

Neutralization. k values. The same hyperimmune guinea pig serum was tested for *k* values (7), using a dilution of 1:160 which neutralized 95–98% of 6.0×10^5 input virus in 15 min at 37°.

Results. Human fibroblasts were chosen for plaque assay of herpesvirus when these cells were found to yield pfu values 1 $\log_{10}$ greater than those found with M-HeLa cells (Table I). Comparable assays were obtained with four strains of fibroblasts and all herpesvirus strains tested plaqued equally well on fibroblasts. Although pock size on the chorioallantoic membrane has been used to differentiate Type 1 from Type 2 herpesvirus strains (8), plaque size on fibrobasts did not vary with type or strain. Plaques produced by the Type 1 strains UW-165, UW-168, E-277, and E-115 could not be distinguished from plaques produced by Type 2 UW-268, E-304, and E-326.

Agarose overlay was preferred to methylcellulose (9), not only because agarose provided a firmer overlay with less opportunity for dispersal of virus within the overlay, but also because plaques under agarose were larger and more discrete than those under methylcellulose. Neither regular agar nor Noble agar were adequate substitutes for agarose.

Cooper (1) discussed six criteria which a plaque assay should fulfill to be considered a useful technique. His criteria were applied to this assay with the following results.

(i) Relation of plaques to inocula. No "plaques" were seen in the absence of viral inocula. All of the fibroblast strains provided good confluent monolayers without interruptions which might be confused with plaques. Plaques due to virus were completely clear areas from 1 to 2 mm in diameter (Fig. 1).

(ii) Reisolation of virus. Virus was readily reisolated from plaques, but no virus was recovered from areas of the plates without plaques. Plaques of UW-168 yielded 10^5–10^6 tissue culture infectious doses/plaque.

(iii) Linearity. Among these criteria, the most important requires that plaque count be proportional to virus concentration, *i.e.*,

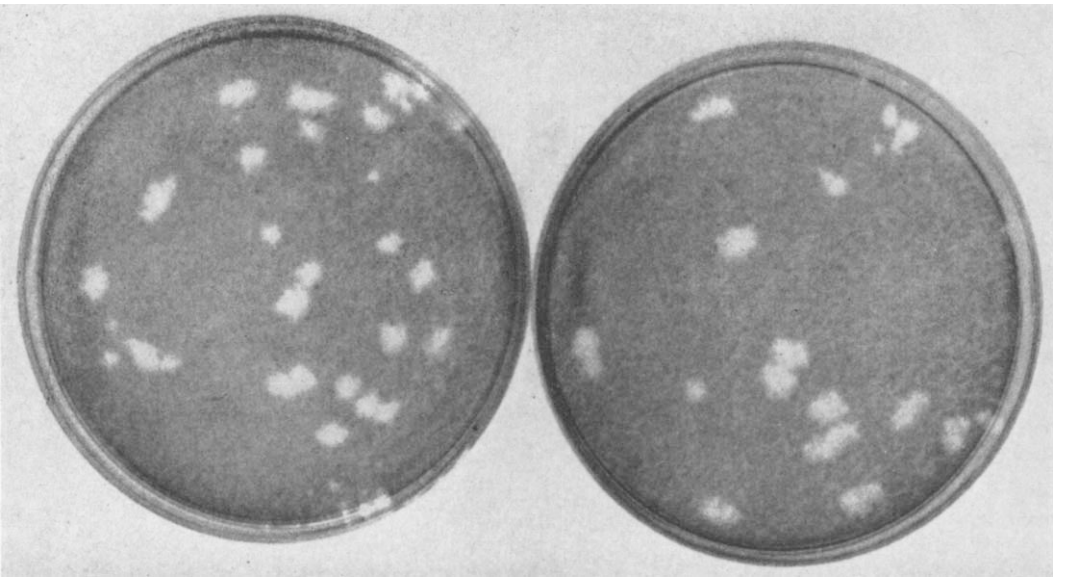

FIG. 1. Photograph of plaques produced by herpesvirus UW-168 on monolayers of human embryonic tonsil fibroblasts under agarose overlay medium; cells are stained with crystal violet.

that a linear relationship be found between number of plaques and the relative virus content of the inocula. UW-168 was assayed in HET cells using 10 plates for each concentration of virus, and the results were tested for linearity of the data. The slope obtained from these data is shown in Fig. 2. Student's t test was used to determine whether the slope of this line is equal to 1 and the intercept equal to 0; $p = 0.4$ and $p = 0.8$, respectively. The distribution of plaques among each group of 10 plates fitted a Poisson distribution when tested by the method of Pettigrew and Mohler (10). Therefore, this assay technique appears to provide linear data within the range of 10–70 plaques/plate.

(iv) Poisson distribution of plaques. Nine replicate assays of UW-168 on HET cells were done, using 10 plates/assay for a final dilution of virus yielding about 30 plaques/plate. Each assay was begun independently from stock virus. The results are shown in Table II. The total number of plaques found in each set of assay plates was homogeneous between assays, as was the distribution of plaques among plates within each assay. The coefficient of variation of replicate assays was 6.4%.

The method of Pettigrew and Mohler (10), used to determine homogeneity among the counts of the linearity data, was also applied to the data from 43 routine assays with 4, 5, or 10 plates/10-fold dilution of virus. No significant lack of homogeneity in the distribution of plaques among plates ($p = 0.05$ level) was found in 41 assays. Therefore, plaques appeared to be distributed among cell cultures of one batch according to a Poisson distribution.

TABLE II. Replicate Assays of Herpesvirus Strain UW-168.

Assay	Mean pfu[a]	p[b]
1	32.9	0.57
2	35.1	0.42
3	37.0	0.01
4	34.3	0.32
5	34.8	0.40
6	32.7	0.99
7	30.3	0.56
8	34.9	0.03
9	30.8	0.24
Total	33.6	0.17

[a] Mean number of plaques per plate for each 10-plate assay.

[b] Probability of observed or more extreme result, assuming a homogeneous distribution of plaques among plates.

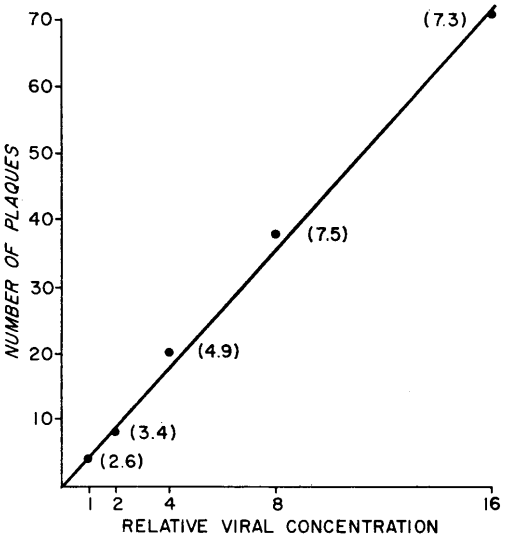

FIG. 2. Relation of mean number of plaques per plate to relative virus concentration: 10 monolayers of HET cells were inoculated with 0.1 ml of each virus dilution, overlaid with agarose medium, and incubated at 37° in 2.5% CO_2 for 96 hr. The monolayers were fixed with formalin, the overlays removed and the cells stained with crystal violet. The numbers in parentheses represent the standard deviation of each set of 10 plates.

(v) Serum neutralization. The UW-165 and UW-168 were neutralized with antiherpes guinea pig serum. This serum had a k value of 33 for UW-165 and of 40 for UW-168, suggesting that they are antigenically related.

The plaque reduction data for UW-168 is shown in Table III. A serum dilution of 1:640 reduced the plaque titer approximately 50% and a dilution of 1:320 reduced the titer by more than 1 $\log_{10}$. Normal guinea pig serum did not inhibit plaque formation by UW-168, nor was Type 1 poliovirus neutralized by the antiherpes serum in comparable control tests. Thus plaque initiation was inhibited by low concentrations of specific antiserum which did not inhibit another virus growing in the same cell system.

(vi) Assay of a preserved stock. Repeated assays of a UW-168 stock held at —80° gave comparable titers with seven different lots of HET cells over a period of 143 days (Table IV). Thus, it was shown that repeated assays of the same virus stock gave essentially the same titer in different batches of cells.

TABLE IV. Repeated Assays of a Preserved Herpesvirus Stock.

Storage at —80° (days)	(pfu/ml)[a]	HET cell lot
60	1.0×10^7	A-16[b]
63	1.0×10^7	A-16
70	1.5×10^7	A-17
104	1.4×10^7	B-12
110	1.6×10^7	B-12
145	1.0×10^7	B-16
181	9.1×10^6	B-15
195	1.0×10^7	C-12
203	1.7×10^7	C-13

[a] Plaque-forming units per ml.

[b] Each lot started from a low-passage stock supplied by Flow Laboratories.

Discussion. Human fibroblasts appear to offer a more sensitive host for plaque assay of herpesvirus than continuous cell lines, since either fibroblast-grown or HeLa-grown stocks

TABLE III. Neutralization of Herpesvirus UW-168 by Hyperimmune Guinea Pig Antiserum.

Virus inocula[a]	Reciprocal of serum dilution 80	160	320	640	Control titration[b]
UW-168, 10^{-3}	2[c]	41	TNTC[d]	TNTC	TNTC
10^{-4}	0	5	73	TNTC	TNTC
10^{-5}	0	0	8	50	91
Poliovirus, $10^{-3.5}$	48	—	—	—	53

[a] Final dilution from a UW-168 virus stock of ca. 10^7 pfu/ml or from a poliovirus stock of ca. 10^6 pfu/ml.

[b] Virus with normal guinea pig serum.

[c] Mean number of plaques per plate.

[d] Too numerous to count.

of virus showed significantly higher titers when assayed on fibroblastic, rather than HeLa, McCoy, or L cells. Garabedian and Scott (9) found primary rabbit kidney cells yielded significantly higher plaque titers than did L cells, but the plaques were indistinct and difficult to count. Plaques on human fibroblasts were large and easy to count. They were completely clear, without the center staining areas described for other cells (9, 11). Plaques on M-HeLa were much smaller than those on fibroblasts and no plaques were obtained from inocula of 10^5 pfu on HeLa-229, a subline shown to be less sensi-contain two unesterified tyrosine residues (4, Plaque count did not appear to vary with these fibroblast strains, although plaque size did. Plaques on WI-38 were smaller than those on HET, even though larger than those found on any continuous cell line.

Human embryonic fibroblasts are easy to obtain commercially and relatively inexpensive to maintain over numerous generations. Although primary cell cultures may be equally sensitive, they do not share this practical advantage.

The use of agarose in overlay media has several advantages. Agarose overlays are easier to prepare and remove from plates than methylcellulose overlays. Since agarose provides a firm overlay, it does not require the addition of specific antiserum to prevent viral spread or examination at an early time, preceding the development of secondary plaques, as do liquid overlays (12). Although they were not as large as those found after 6-days incubation and two sequential overlays by Daniel and Melendez (2), plaques under one agarose overlay were plainly visible to the naked eye and countable after 3-days incubation. Incubation beyond 96 hr did not increase either plaque size or number.

The addition of magnesium sulfate to give a final concentration of 30 m*M* in overlays (4) reduced the virus titer 1 $\log_{10}$ or more in either HeLa or HET cell assays. This is in accord with the reported sensitivity of herpesvirus to low salt concentrations (13). Whereas regular or Noble agar required the addition of DEAE-dextran to overlays for production of any visible plaques, agarose did not require this additive. The simplicity, sensitivity, and reproducibility of the assay technique described here suggest that it is a useful method for quantitation of herpesvirus.

We would like to thank Dr. Blair E. Bennett for assistance in the statistical analysis of the data; Dr. James Gale for help with serologic studies; Dr. George E. Kenny for culturing cells and virus for mycoplasmata; and Miss Patricia Skahen for technical assistance.

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Received Feb. 12, 1969. P.S.E.B.M., 1969, Vol. 131.