Plaque Assay of Cytomegalovirus Strains of Human Origin (35031)

BERTTINA B. WENTWORTH AND LINDA FRENCH
(Introduced by J. T. Grayston)
Department of Preventive Medicine, School of Medicine, University of Washington, Seattle, Washington 98105

The use of human fibroblasts with an agarose overlay medium was found to be a reliable and practical method for the plaque assay of *Herpesvirus hominis* (1). This technique has now been modified for use in assaying cytomegalovirus (CMV) strains of human origin. Two methods for the quantitation of cytomegalovirus have been described previously, in addition to the traditional determination of 50% tissue culture infectivity doses by tube dilution. The first of these, the microscopic counting of infected cells on coverslip preparations after either immunofluorescent (2) or Giemsa (3) staining had obvious limitations and technical difficulties. Plummer and Benyesh-Melnick (4) then described a plaque assay technique using methyl cellulose in the overlay medium. Since agarose overlays are more easily handled than methyl cellulose and afford a firm overlay to prevent viral spread, we investigated the use of an assay with two sequential agarose overlays. The criteria for a useful plaque assay described by Cooper (5) were used to evaluate this technique.

Materials and Methods. Cells. The FT line of human fibroblasts was derived in our laboratory from embryonic tonsillar tissue. Routine cultures of this cell line have never yielded any mycoplasmata when incubated both aerobically and anaerobically during the period of the study.

Media. Cells were grown in Eagle's minimum essential medium with 10% fetal calf serum, 6.6 m*M* sodium bicarbonate and appropriate antibiotics (1). Virus was diluted in maintenance medium containing 2% "GG-free" calf serum in place of fetal calf serum (MEM2). Overlay medium consisted of equal parts of double strength MEM2 and 0.6% agarose (SeaKem, Bausch and Lomb Inc., Rochester, N.Y.) in distilled water. The sodium bicarbonate was increased to a final concentration of 8.0 m*M* in the complete medium.

Virus strains. Four CMV strains isolated in our laboratory (UW-1, UW-2, GU-569, G-35) and 2 strains isolated in Taiwan (TW-008, TW-087) were used. UW-1 and UW-2 were obtained from neonatal cases of congenital cytomegalic inclusion disease and GU-569 from an asymptomatic newborn infant: G-35, TW-008, and TW-087 were isolated from pregnant females (6, 7). The AD169 strain used was kindly supplied by Dr. Helen Casey, National Communicable Disease Center.

The L.Sc. strain of type 1 poliovirus, strain UW-168 type 1 herpesvirus and UW-268 type 2 herpesvirus (1) were used in the neutralization studies.

Virus stocks consisted of extracellular virus from infected monolayers. Monolayers infected with CMV were held for 4–6 days at 37° after the development of extensive cytopathology involving at least 90% of the cell layer. The supernatant fluids were removed and filtered through Millipore filters of 0.45-μ porosity. Aliquots were stored at −80° without the addition of any preservative. Stocks prepared in this manner had titers of 10^5–10^8 pfu/ml. All virus stocks were cultured for mycoplasmata before storage; none were found contaminated.

Plaque assay. Monolayers were prepared by seeding 60-mm plastic tissue culture dishes with 10^6 FT cells in 5 ml of growth medium. After overnight incubation at 37° in 2.5% CO_2 in air, the growth medium was removed and each plate was inoculated with

0.1 ml of suitably diluted virus. Inocula were adsorbed for 30 min at room temperature before 5 ml of overlay medium was added to each plate, since counts were not increased by prolonged adsorption, up to overnight at 37°. Plates were incubated at 37° in 2.5% CO_2 for 7 days, then a second overlay of 5 ml of overlay medium was added over the first overlay. The plates were incubated for a further 7 days under the same conditions. Two ml of 10% formalin was added to each plate to fix the monolayers. After 10 min at room temperature, the overlays were discarded, the monolayers were washed with tap water and stained with 0.03% aqueous methylene blue for 1–2 min. Plaques were counted with the aid of a stereoscopic microscope providing magnification of 7× to 42× (Stereo-Star/Zoom microscope, American Optical Corp., Buffalo, N.Y.), which greatly facilitated accurate counting.

For reisolation of virus, plaques were removed from plates with capillary pipettes before the addition of formalin and assayed by a tube-dilution technique.

Neutralization. Plaque reduction. Dilution of a hyperimmune guinea pig serum were mixed with suitable dilutions of virus stock and the mixtures were allowed to stand for 1 hr at room temperature before being assayed for residual virus by the plaque technique. The hyperimmune guinea pig serum was obtained after two intramuscular injections of homogenized cells infected with the UW-1 strain. Preimmunization guinea pig serum was used as control.

Neutralization. k values. k values for the hyperimmune guinea pig serum were estimated (8), using a dilution of 1:50 which neutralized 98–99% of 3.5×10^5 input UW-1 virus in 10 min at 37° for the homologous system.

Results. The plaque assay was evaluated by the criteria proposed by Cooper (5) for determining satisfactory plaque techniques with the following results.

(i) *Relation of plaques to inocula.* Foci of cytopathology were easily distinguished from the normal fibroblast cell layer and no artifacts which might be confused with the deeply staining plaque areas were seen. Plaques ranged from ca. 0.5 to 1.5 mm in diameter (Fig. 1a). Larger plaques often had clear central zones devoid of cells while smaller plaques consisted entirely of darkly stained cells (Fig. 1b). No foci were seen in the absence of viral inocula.

(ii) *Reisolation of virus.* Virus could be readily recovered from plaques when plaque material was transferred to 1 ml of medium and assayed for infectivity. No evidence of infectivity was obtained when areas devoid of plaques were tested in a like manner. UW-1 plaques yielded 10^2–10^3 tissue culture infectious doses. Disruption of infected cells in plaque material by homogenization did not increase the recovery of viral infectivity.

(iii) *Linearity.* Plaque count was found to be proportional to virus concentration such that an approximately linear relationship existed between the number of plaques and the relative virus content of the inocula. UW-1 virus was assayed on FT cells, using 10 plates for each concentration of virus in 2 separate experiments. The results shown in Fig. 2 were tested for linearity of the data. Student's t test was used to determine whether the slope of the lines was equal to 1 and whether the intercepts were equal to 0. For Expt. 1, $p = 0.99$ and 0.78, respectively, and for Expt. 2, $p = 0.73$ and 0.72. This assay thus appears to provide linear data with a range of 10–100 plaques/plate.

(iv) *Poisson distribution of plaques.* Several experiments consisting of 9 replicate assays of a UW-1 stock on FT cells were done, using 6–10 plates/assay for a final dilution of virus yielding 50–70 plaques/plate. In none of these experiments were the results from replicate plates sufficiently homogeneous to be represented adequately by a Poisson distribution. Variation between the means of replicate assays was less, however, than the variation between replicate plates within assays and the means were not significantly different. Coefficient of variation within replicate assays varied from 9 to 27% with an average of 19%. The coefficient of variation for the means of the replicate assays was 8%, so that the titers obtained for 9 replicate

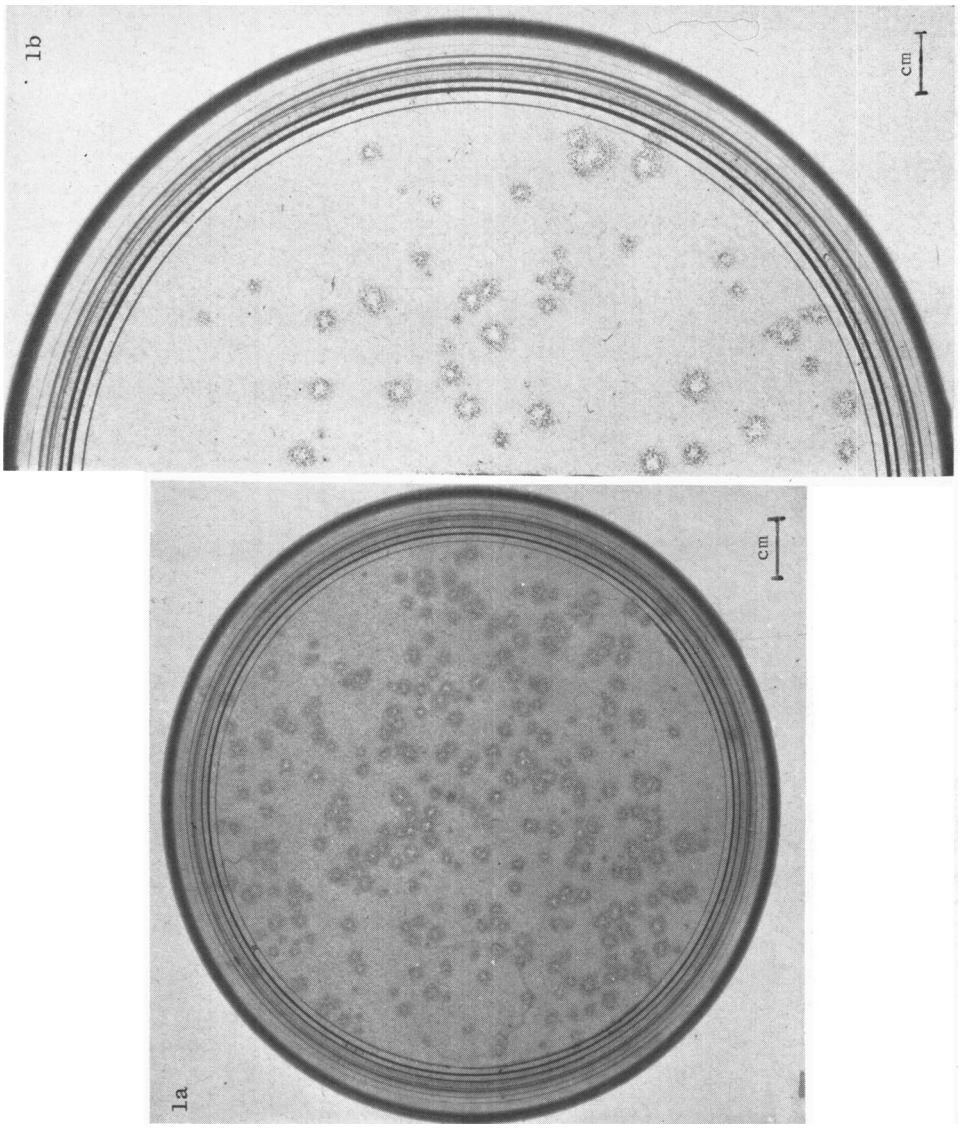

FIG. 1. Photograph of plaques produced by human CMV strains UW-1 and G-35 in monolayers of human embryonic fibroblasts under agarose overlay medium; cells stained with methylene blue: (a) UW-1, low magnification; (b) G-35, high magnification.

assays fell between 6.6×10^6 and 8.0×10^6 (Table I). In spite of the variation between replicate plates, reproducible titers can be obtained from repeated assays, whose means are homogeneously distributed.

(v) *Serum neutralization.* A hyperimmune guinea pig serum was used to neutralize UW-1 in a plaque reduction test where type 1 and type 2 herpesviruses and type 1 poliovirus were used as controls. The results are shown in Table II. A serum dilution of 1:128 reduced the UW-1 titer approximately 90%. A dilution of 1:16 did not neutralize either the poliovirus or the type 1 herpesvirus, but did produce more than a 50% reduction of the type 2 herpesvirus. This serum could not be tested at lower dilutions, since it was toxic for the cells when diluted 1:8. Preimmune serum from the same guinea pig did not inhibit plaque formation by either the UW-1 CMV or the type 2 herpesvirus.

The k values of the hyperimmune guinea

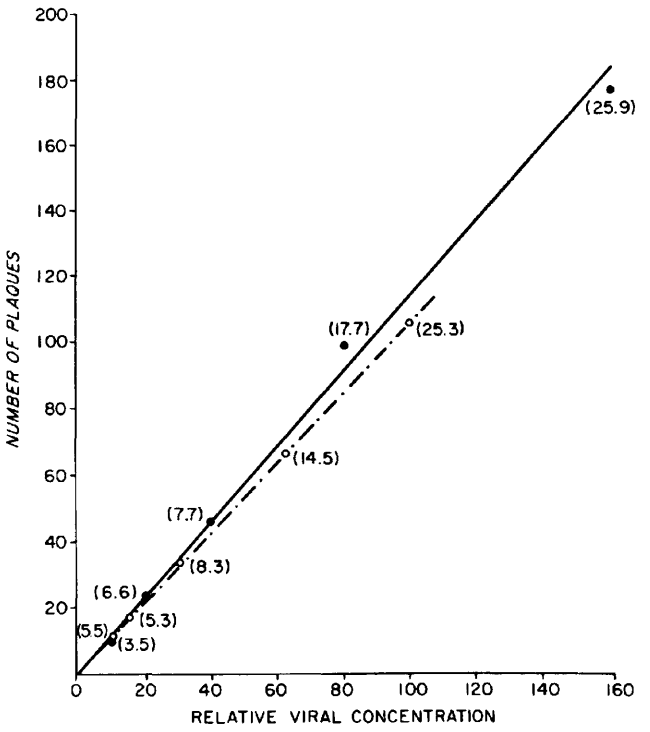

FIG. 2. Relation of mean number of plaques per plate to relative virus concentration: 10 monolayers of FT cells were inoculated with 0.1 ml of each virus dilution, overlaid with agarose medium after 0 and 7 days' incubation at 37° in 2.5% CO_2. After a further 7 days of incubation, the monolayers were fixed with formalin and stained with methylene blue after removal of overlays. The numbers in parentheses represent the standard deviation of each set of 10 plates. Expt. 1 (—); Expt. 2 (—.—).

pig serum were estimated, using CMV strains UW-1, UW-2, AD169, and the type 2 herpesvirus. The results shown in Table III suggest that UW-1 and UW-2 are closely related antigenically and that neither are closely related to the AD169 strain. There was some neutralization of the type 2 herpesvirus, but the k was less than 1.

From these results it appears that plaque initiation by a CMV strain was inhibited by low concentrations of an antiserum which did not inhibit other viruses in the same system.

(vi) *Assay of preserved stocks.* Repeated assays of preserved CMV stocks held at —80° over periods up to 230 days of storage gave comparable titers with different lots of cells by the plaque technique. Some loss of viability may have occurred with time, but no significant changes were observed. Observations on 7 stock strains are shown in Table IV. Three strains of FT cells from a single frozen stock were used in passages 8 to 26 to obtain these results.

TABLE I. Mean Number of Plaques per Plate for Nine Replicate Assays of UW-1 on FT Cells.

Assay	Mean pfu[a]
1	66.8
2	67.5
3	77.3
4	66.8
5	79.8
6	68.0
7	65.5
8	74.3
9	78.0
Coefficient of variation	8%
SD	5.7

[a] Plaque-forming units per ml.

TABLE II. Plaque Reduction by CMV UW-1 Hyperimmune Guinea Pig Antiserum.

Virus inocula[a]	Reciprocal of serum dilution							Control titration[b]
	16	32	64	128	256	512	1024	
CMV UW-1 10^{-5}	0[c]	0	8	51	TNTC[d]	TNTC	TNTC	TNTC
10^{-6}	0	0	1	6	20	31	44	52
Poliovirus 10^{-6}	20	32	—	—	—	—	—	25
Herpesvirus-1[e] 10^{-5}	20	32	26	—	—	—	—	28
Herpesvirus-2[e] 10^{-4}	18	41	50	67	—	—	—	70

[a] Final dilution from CMV and poliovirus stocks of ca. 10^7 pfu/ml or from herpesvirus stocks of ca. 10^9 pfu/ml.

[b] Virus with preimmunization guinea pig serum and/or MEM2.

[c] Mean number of plaques per plate.

[d] Too numerous to count.

[e] Herpesvirus type.

Plaque titers were compared with the results obtained from tube-dilution titrations. Our tube-dilution titrations are read after 5 weeks' incubation since this was found to be the optimum time (6). In most cases the plaque titers agreed satisfactorily with the infectious doses measured by tube-dilution titrations, both falling within the same $\log_{10}$ (Table IV). Since the plaques are counted after only 2 weeks' incubation, tube-dilution titers at the end of 2 weeks were compared with plaque titers. Where the 2 weeks' readings differed from the 5 weeks' readings, plaque counts were always higher than the tube-dilution results read at 2 weeks.

From these and similar data it was concluded that repeated assays of preserved stocks by this plaque technique yielded essentially the same titers in different batches of cells.

Discussion. A plaque assay employing two sequential agarose overlays appears to provide a simple, reliable method for quantitation of CMV strains. This assay satisfied the criteria of Cooper (5). As noted with the plaque assay for *Herpesvirus hominis*, the agarose overlay had several advantages over methyl cellulose overlays. Not only were agarose overlays easier to prepare and remove from plates than methyl cellulose, but CMV plaques were more discrete and easier to count under agarose. Neither the strain of fetal tonsil cells used nor the number of prior passages appeared to influence plaque production, although the number of cells used to initiate monolayers did. Plaque counts were sometimes reduced if less than 10^6 cells were used to seed plates. Methylene blue staining was found to be superior to crystal violet (1), methyl red (9) or INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride) (10). Crystal violet gave too intense a color, while the vital staining gave too little contrast.

TABLE III. Neutralization by CMV UW-1 Hyperimmune Guinea Pig Antiserum: k Values.

Virus	k value[a]
CMV UW-1	21
CMV UW-2	27
CMV AD169	7
Type 2 Herpesvirus	0.5

[a] Calculated from the neutralization of ca. 10^5 input virus in 10 min at 37°.

The use of a practical and accurate method for quantitation of CMV made more sophisticated neutralization studies possible. The preliminary results of k values on one hyperimmune serum suggested that antigenic variation exists among CMV strains. The neutralization results also indicated a minor cross-reaction between CMV and type 2 herpesvirus. Although it is impossible to rule out the possibility of exposure of the animal to herpesvirus during the immunization process, no source of such stimulation was apparent. Since type 2 herpesvirus can be differentiated from type 1 by several biological markers (11), it may fall into an intermediate class which has minor cross-reactivity with CMV,

TABLE IV. Repeated Assays of Preserved Cytomegalovirus Stocks.

Virus stock	Storage (days)	pfu[a] titers	TCD_{50}[b] titers		FT cell lot[c]
			5 weeks	2 weeks	
UW-1	1	—	5×10^5	1×10^5	A-17
	73	2.4×10^6	—	—	B-13
	123	1.1×10^6	—	—	B-24
	149	1.4×10^6	—	—	C-18
	150	1.2×10^6	—	—	C-18
	203	—	1×10^7	5×10^5	C-11
	207	1.4×10^6	—	—	C-24
UW-2	1	—	5×10^7	5×10^7	A-15
	47	6.9×10^6	—	—	B-17
	64	1.0×10^7	—	—	C-13
	85	5.4×10^6	—	—	C-12
	212	—	5×10^7	5×10^7	C-12
	330	5.9×10^6	—	—	C-26
G-35	1	—	5×10^6	5×10^6	A-17
	80	6.5×10^6	—	—	B-13
	101	3.4×10^7	—	—	B-12
GU-569	52	1.4×10^6	—	—	B-13
	73	1.5×10^6	—	—	B-12
	99	—	2×10^6	1×10^6	B-14
TW-008	62	2.3×10^5	—	—	B-13
	83	2.1×10^5	—	—	B-12
	110	2.6×10^5	—	—	B-14
	115	—	4.7×10^5	4.7×10^5	B-15
TW-087	7	—	1×10^6	5×10^5	A- 8
	59	7.2×10^5	—	—	B-13
	80	7.2×10^5	—	—	B-12
AD169	1	—	1×10^7	1×10^7	A-15
	64	1.7×10^7	—	—	B-13
	85	9.2×10^6	—	—	B-12
	330	3.4×10^6	—	—	B-26

[a] Plaque-forming units per ml.

[b] 50% Tissue culture infectious doses per ml after either 2 or 5 weeks' incubation.

[c] Cell subline and prior number of passages.

while sharing major antigens with type 1 herpesvirus.

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