

Microculture Plaque Assay for Human and Simian Cytomegaloviruses

SHUNZO CHIBA,¹ ROBERT L. STRIKER, JR., AND MATILDA BENYESH-MELNICK

Department of Virology and Epidemiology, Baylor College of Medicine, Houston, Texas 77025

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The plaque assay for human and simian cytomegaloviruses routinely carried out in 60-mm petri dishes (macrocultures) has been adapted for use in microcultures in flat-bottom 16-mm circular wells of disposable plastic trays. Virus titrations and serum neutralization assays carried out in microcultures yielded reproducible results that were identical to those obtained in macrocultures.

Two plaque assay procedures for human cytomegaloviruses (CMV) have been described, one employing methyl cellulose (7, 9) and the other agarose (11) in the overlay medium. Both methods have made use of cultures in 60-mm petri dishes (macrocultures). In view of the finding that human (3, 7, 9, 11) and simian (3) strains of CMV produce minute plaques, detectable under a low-power microscope, attempts were made to adapt the macroculture plaque assay (7, 9) for use in microcultures. This paper describes the development of a microculture plaque assay for human and simian CMV which proved to be as reliable and quantitative as but much more economical than the macroculture test previously used (7, 9). The new test can be used for virus titrations and for plaque reduction neutralization tests.

MATERIALS AND METHODS

Cell cultures. Human embryonic lung (HEL) fibroblasts, propagated as previously described (9) were used. Cells were grown in Eagle minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 0.075% NaHCO₃ (for cells in stoppered vessels) or 0.225% NaHCO₃ (for cells kept in a 5% CO₂ atmosphere).

Plastic tissue culture trays [Linbro Disposotray, model FB16-24 TC, size 4 by 6 inch (approx. 10 by 15 cm)] with 24 flat-bottom circular wells (16-mm diameter, 3.5-ml capacity) were used for the preparation of microcultures, and 60-mm plastic petri dishes were used for the preparation of macrocultures. Freshly trypsinized HEL cells were washed once with MEM, resuspended in growth medium, and seeded at a concentration of 2×10^6 cells (in 5 ml) per macroculture and of 2×10^5 cells (in 1 ml) per microculture. All cultures were incubated in a

humidified CO₂ incubator at 37 C for 72 hr, at which time complete monolayers were obtained.

Viruses and antisera. Two human CMV strains, AD169 (8) and C87 (2), and one simian strain, GR2757, isolated from an African green monkey (3) were used. The viruses were kept in passage in HEL fibroblasts as described elsewhere (3, 9). Cell-free virus stocks were prepared as described previously (1, 3, 9) and stored at -90 C in the presence of 35% sorbitol (9) until used.

Specific hyperimmune goat sera to the three CMV strains prepared as described earlier (5) were used for neutralization tests. Sera were stored at -20 C and inactivated at 56 C for 30 min before use.

Plaque assay. The regular plaque assay for CMV (7, 9) was modified for use in microcultures by reducing the amount of inoculum and of overlay medium. Virus dilutions were made in bicarbonate-free MEM with 5% heat-inactivated FBS. When complete monolayers were formed, the medium was aspirated from the cultures. Each virus dilution was inoculated into replicate macrocultures and microcultures in 0.2 ml and 0.05 ml, respectively. After 1 hr of adsorption at 37 C, 7-ml and 1-ml amounts, respectively, of an overlay containing 2% methyl cellulose (9) were added to each macro- and microculture; the same procedure was repeated 7 days later with an overlay containing 1.4% methyl cellulose (9). At day 14, plaques were counted in all cultures as described earlier (7, 9).

Neutralization test. The detailed procedures for neutralization tests carried out in the presence or absence of complement have been described (4, 5). Briefly, the test employed fourfold dilutions of heat-inactivated test serum, complement at a dilution containing 5 hemolytic units, and virus diluted to contain 1,200 plaque-forming units (PFU) per 0.2 ml. Bicarbonate-free MEM with 5% heat-inactivated FBS was used as diluent for all reagents. Serum, complement, and virus (for tests in the presence of complement), or serum, diluent, and virus (for tests in the absence of complement) were mixed at a ratio of 2:1:1. For virus controls, diluent was used to substitute for either serum or serum and complement. After incubation for 1 hr at 37 C, residual infectivity

¹On leave from Sapporo Medical College, Sapporo, Japan, as a trainee of the United States-Japan Cooperative Medical Science Program.

was determined in both macro- and microcultures; 0.2 ml or 0.05 ml, respectively, of each reaction mixture was inoculated in parallel to each of two petri dishes or of two wells in the microculture trays. Antibody titers were expressed as the reciprocal of the final serum dilution exhibiting 60% plaque reduction (3).

RESULTS

Comparative plaque assays in macro- and microcultures. In preliminary experiments, CMV titrations were carried out in parallel in macrocultures, using the standard inoculum of 0.2 ml, and in microcultures, using an inoculum of 0.05 or 0.1 ml. Whereas the microcultures inoculated with 0.05 ml of a certain virus dilution consistently yielded approximately 25% of the number of plaques in the macrocultures inoculated with 0.2 ml of the same virus dilution, those inoculated with 0.1 ml yielded plaque counts below the expected, indicating a less efficient virus adsorption during the 1 hr of incubation used. Thus, an inoculum size of 0.05 ml (which can be easily attained by delivering one drop with a 1-ml pipette) was used for all microculture plaque assays described hereafter.

Representative dose-response curves for strain C87 given in Fig. 1 indicated an excellent agreement between the results obtained by the two methods. Table 1 shows the results of three consecutive parallel virus titrations with strains C87, AD169, and GR2757. There was no appreciable difference in titer observed between the two assay procedures, and reproducible results were obtained by both methods.

Comparative neutralization tests in macro- and microcultures. Since microcultures could be used as efficiently as macrocultures for quantitative plaque assays, attempts were made to determine their applicability to plaque reduction neutralization tests with hyperimmune sera. Neutralization tests in the presence or in the absence of complement were performed as described above. Repeat cross-neutralization tests using strains C87, AD169, and GR2757 and the respective hyperimmune goat sera (Table 2) revealed a good correlation between the two methods of assay used. As we have reported earlier using the same goat antisera (5), there was complete cross-neutralization between human strains C87 and AD169 and a lack of cross-reactivity between them and the simian virus GR2757. Also, as we have shown earlier (4, 5), the hyperimmune neutralizing antibody to human CMV was predominantly complement-dependent, whereas antibody to simian CMV was complement-independent.

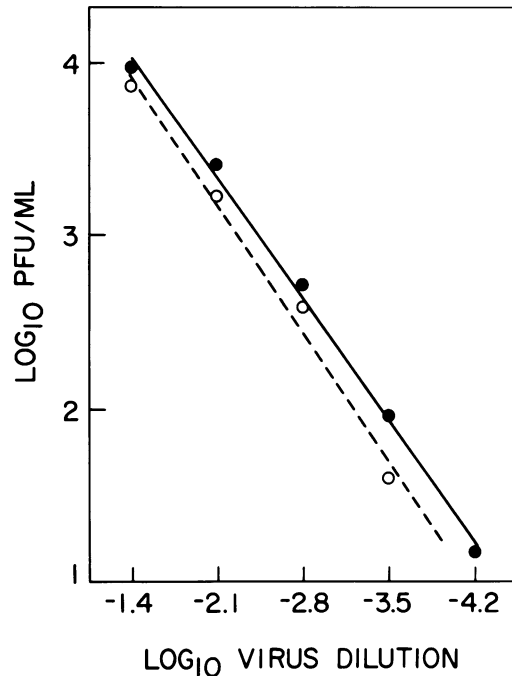


FIG. 1. Dose-response curves for CMV strain C87. Fivefold virus dilutions were assayed in 0.2- and 0.05-ml amounts in macrocultures (●) and microcultures (○), respectively, and the number of plaque-forming units (PFU) per ml of each virus dilution was determined.

DISCUSSION

The results of the present study indicate that the microcultures described can be used for reliable and quantitative plaque assays with human and simian CMV. The microculture assay offers a great advantage over the regular test in 60-mm petri dishes in that it is more economical not only in reagents but also in time, since it permits a large number of determinations in one plate. Except for reducing the volume of inoculum and overlay, no further changes had to be introduced to the manner in which virus titration (3, 7, 9) or serum neutralization tests (4, 5) have been conducted in this laboratory.

Another advantage of the microcultures is that reliable results can be obtained even though fewer plaques are counted than with the macrocultures, the morphology and size of plaques (7) being the same in both types of cultures. As with macrocultures, there was little or no variation in plaque counts between replicate microcultures within a test. Thus, two replicate microcultures in the wells of the trays can be used as reliably as two cultures in 60-mm petri dishes for each virus dilution or

TABLE 1. Results of parallel virus titrations in macro- and microcultures

Virus strains	Plaque-forming units per ml in:					
	Macrocultures			Microcultures		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
C87	2.4×10^6	2.7×10^6	1.8×10^6	1.4×10^5	1.4×10^5	1.7×10^5
AD169	4.8×10^6	4.6×10^6	3.6×10^6	4.0×10^5	3.6×10^5	2.5×10^5
GR2757	2.1×10^7	1.7×10^7	1.6×10^7	1.2×10^7	1.0×10^7	1.1×10^7

TABLE 2. Results of parallel neutralization tests in macro- and microcultures

Hyperimmune goat antiserum to strain	Expt no.	Cultures used	Neutralizing antibody titers ^a to strains:					
			C87		AD169		GR2757	
			-Complement ^b	+Complement	-Complement	+Complement	-Complement	+Complement
C87 (human)	1	Macro	80	1600	40	1650	<16	<16
		Micro	70	1650	90	2400	<16	<16
	2	Macro	140	1900	40	3100	<16	<16
		Micro	60	2000	50	3100	<16	<16
AD169 (human)	1	Macro	380	2000	140	2200	<16	<16
		Micro	320	2200	350	3000	<16	<16
	2	Macro	370	2900	560	3100	<16	<16
		Micro	260	2600	600	4096	<16	<16
GR2757 (simian)	1	Macro	<16	<16	<16	<16	610	950
		Micro	<16	<16	<16	<16	640	640
	2	Macro	<16	<16	<16	<16	420	500
		Micro	<16	<16	<16	<16	510	510

^a Reciprocal of highest final serum dilution giving 60% plaque reduction.

^b -Complement = test in the absence of complement; +complement = test in the presence of 5 to 10 hemolytic units of complement.

reaction mixture in a test. It should be noted that as many as 250 to 300 plaques can be reliably counted within a microculture; for plaque reduction neutralization tests, counts of 60 to 100 are optimal.

A miniculture plaque assay for murine CMV has been reported recently (6) that employs ring cultures on microscope slides. The present method is of greater advantage in that it uses commercially available panels which are much easier to handle. Larger Disposotrays with 96 wells have been used by others for a 5- to 6-day plaque reduction neutralization test with the Tacaribe virus group (10). Since the assay of human and simian CMV requires an incubation of 14 days and the application of a double methyl cellulose overlay (7, 9), these larger panels could not be used particularly because of the increased frequency of bacterial or fungal contamination, or both; this problem was not encountered with the 24-well disposotray used.

In view of the results of the present study, the microcultures described are now being routinely used in this laboratory for all human and simian CMV assays.

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