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Plaque Reduction Neutralization Test for Human Cytomegalovirus Based upon Enhanced Uptake of Neutral Red by Virus-Infected Cells

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Foci of cells infected with human cytomegalovirus were noted to stain more intensely than uninfected cells with neutral red, and this provided the basis for development of a plaque assay and plaque reduction neutralization test for cytomegalovirus. Plaques demonstrable by neutral red staining could be counted at 8 days after infection; thus, results could be obtained earlier than for plaque assay systems based upon the viral cytopathic effect, and fewer manipulations were required for staining cell monolayers to demonstrate plaques. Certain variables affecting plaque size and numbers and antibody titers were defined. Addition of fresh guinea pig complement to the reaction mixtures markedly enhanced cytomegalovirus-neutralizing antibody titers of hyperimmune animal sera, but titers of human sera were enhanced only two- or fourfold.

Several plaque assays for human cytomegalovirus (CMV), based either upon the viral cytopathic effect (1, 9, 12) or fluorescent antibody staining of infected cells (11), have been described in recent years, and these have been successfully applied to the assay of CMV-neutralizing antibody. Disadvantages of plaque assays based upon the cytopathic effect of CMV are the requirement for an incubation period of 14 days or longer and the need to remove the overlays before staining the cell monolayers. Assays based upon fluorescent antibody staining of infected cells utilize only a 3-day incubation period (11), but they require special equipment, time-consuming microscopic observation, and a reliable source of antisera that give specific staining for CMV.

It was noted in this laboratory that, within a few days after inoculation, foci of CMV-infected cells stain more intensely with neutral red than do surrounding uninfected cells in the monolayer, and this provided the basis for the development of the plaque assay and plaque reduction neutralization test for CMV described in this report.

MATERIALS AND METHODS

Cell cultures. Tests were conducted in lines of human fetal diploid lung cells established by J. H. Schieble of this laboratory. Virus for use in neutralization tests was propagated in these cell lines or in WI-38 cells obtained from Flow Laboratories. Cells were routinely propagated on fortified Eagle minimal essential medium (MEM) (containing two times the standard concentrations of vitamins and amino acids) supplemented with 10% fetal bovine serum.

Virus strains. The AD-169 strain of human CMV was used throughout these studies. High-titered virus was prepared in roller bottles by infecting the cell cultures maintained on 90% fortified Eagle MEM and 10% heated (56°C, 30 min) fetal bovine serum at a multiplicity of ≥1 plaque-forming unit (PFU)/cell. Infected cultures were incubated at 36°C for 9 to 10 days. The cells were then dispersed into the medium by shaking with glass beads, and the material was frozen and thawed three times and then clarified by centrifugation at 8,000 × *g* for 20 min. The clarified fluids were centrifuged at 47,000 × *g* for 60 min to sediment the virus, and the pellets were resuspended in supernatant fluid to 1/20 of the original culture volume. The virus preparations were stored at −70°C. Infectivity titers were $\geq 10^7$ PFU/ml.

Plaque assays were also performed on low-passage-level CMV strains isolated in this laboratory from clinical specimens.

Sera examined. Reference antisera to the AD-169 and C87 strains of human CMV produced in monkeys or goats were obtained from J. L. Melnick. Antiserum to the AD-169 strain was prepared in hamsters by B. Forghani of this laboratory (Forghani, Schmidt, and Lennette, manuscript submitted for publication). Human sera assayed for neutralizing antibody to CMV were from our diagnostic files; most of these were from patients who had shown diagnostically significant increases in titer to CMV complement-fixing (CF) antigen, and some sera were selected on the basis of negative CF reactivity to CMV.

CMV plaque assays and plaque reduction neutralization tests. Plastic plates (wells, 16 mm in diameter) from three different sources, i.e., no. 3008 Multiwell tissue culture plates (Falcon, Oxnard,

Calif.), FB-16-24-TC Multidish Dispo-Trays (Linbro Chemical Co., Van Nuys, Calif.), and no. 3524 Tissue Culture Cluster[24] (Costar, Cambridge, Mass.), were used with satisfactory results. Trypsin-dispersed human fetal diploid lung cells were suspended in growth medium to a concentration of 150,000 cells/ml. The growth medium consisted of 90% fortified Eagle MEM and 10% fetal bovine serum; it was buffered by the addition of 1.5 ml of 8.8% $NaHCO_3$ per 100 ml of medium. A 1-ml amount of the cell suspension was added to each well, and cell monolayers were used after 24 h of incubation at 36°C in a CO_2 incubator. Virus, sera, and guinea pig complement were diluted in Hanks balanced salt solution containing 5% inactivated (56°C, 30 min) fetal bovine serum.

For plaque reduction neutralization tests, serial twofold dilutions of sera (inactivated at 56°C for 30 min) were prepared in 0.2-ml volumes, to which was added 0.1 ml of a 1:8 dilution of fresh guinea pig serum (approximately 10 hemolytic units of complement). In some experiments described below, comparative tests were done using fresh, unheated guinea pig serum and guinea pig serum heated at 56°C for 30 min. Virus diluted to contain approximately 4,000 PFU/ml was then added in a volume of 0.1 ml, and mixtures were incubated at 37°C for varying lengths of time. Based upon experimental results described below, an incubation period of 90 min was adopted for routine use. The serum-virus mixtures were than inoculated onto monolayer cultures in the wells in a volume of 0.1 ml, and the cultures were incubated at 36°C in a CO_2 incubator for 50 to 60 min to permit adsorption of unneutralized virus. The inocula were then removed, and the cell sheets were washed once with 1 ml of diluent and overlaid with 1 ml of serum-free nutrient overlay. This consisted of standard Eagle MEM (prepared without phenol red) supplemented with 0.1% bovine serum albumin, 0.1% yeastolate, and 0.5% ionagar no. 2 (Colab Labs., Inc., Chicago Heights, Ill.) or agarose (SeaKem, Marine Colloids, Inc., Rockland, Me.); it was buffered by the addition of 1.5 ml of 8.8% $NaHCO_3$ per 100 ml of medium. After 7 days of incubation at 36°C in a CO_2 incubator, 0.25 ml of the above medium containing 7% of a 1:1,000 stock solution of neutral red was added to each well, and incubation was continued for an additional day.

Plaques were counted 24 h after the addition of the second overlay with a Wild M5 stereomicroscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland) at ×25 magnification, using transmitted light against a white background (4). A cross-hatched reading plate was used to aid in counting the plaques.

CMV-neutralizing antibody titers were expressed as the highest dilution of serum producing a 50% or greater reduction in plaque count, as compared with the controls in which the test dose of virus was plaqued in the presence of a 1:8 dilution of fresh or heated guinea pig complement, and diluent in lieu of test serum.

RESULTS

Effect of certain variables on CMV plaque size and numbers. The development of foci of CMV-infected cells showing enhanced uptake of neutral red was found to be most pronounced under a serum-free overlay and, therefore, the plaquing medium described above was developed. Figure 1 shows a single focus of CMV-infected cells stained at 7 days after infection and photographed 24 h later. With increased incubation, the dead cells in the center of the CMV-infected foci failed to take the vital stain, whereas the more recently infected cells at the periphery of the foci showed intense staining (see Fig. 2).

Comparative studies showed that the size and number of plaques were comparable under overlays containing either ionagar no. 2 (Colab, Chicago Heights, Ill.) or agarose (SeaKem, Marine Colloids, Inc., Rockland, Me.); however, those under ionagar 2S (marketed as a replacement for ionagar no. 2) were small and difficult to count.

Table 1 shows a comparison of the size and numbers of plaques obtained when the second overlay containing neutral red was added at varying times after infection. In all experiments the plaques were counted 24 h after the addition of the second overlay. Plaques stained at 6 days were smaller and somewhat fewer than those stained at 7 days. Although plaques stained at 11 or 12 days were considerably larger than those stained at 7 days, there was little or no increase in plaque numbers. In the interest of obtaining results earlier, plaques were overlaid routinely at 7 days.

Another variable found to affect plaque counts was the number of human fetal diploid lung cells in the monolayer inoculated with virus. Monolayers produced at 24 h by plating fewer than 150,000 cells/well gave lower plaque counts. However, plaque counts were comparable in monolayers initiated with 150,000 to 300,000 cells. Wentworth and French (12) noted a similar effect of the number of cells plated on plaque counts of CMV.

The plaquing ability of different strains of human CMV was investigated by using five field strains of virus isolated in this laboratory from clinical specimens. The isolates were at passage level 1 to 3, and all produced plaques similar to those produced by the AD-169 strain under identical conditions.

Relationship between plaque numbers and virus concentration. Figure 3 shows the results of three different titrations of CMV stock preparations. Each point on the graph represents the average plaque count on four wells. The plaque counts are seen to vary directly with the dilution of the virus preparations, indicating that each plaque was produced by a single infectious virus particle.

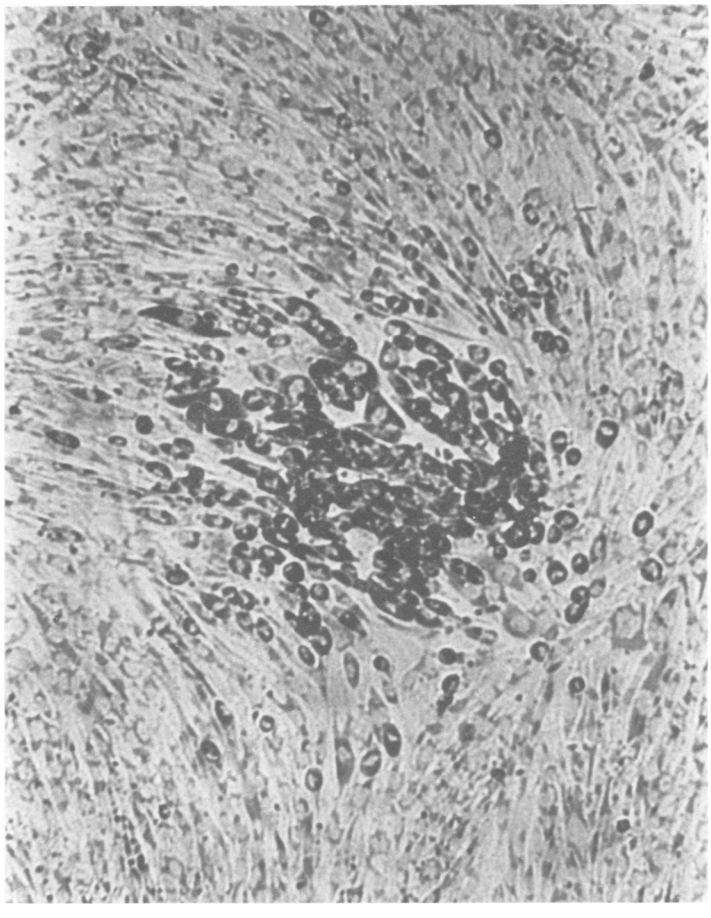

FIG. 1. *Focus of CMV-infected cells showing enhanced staining with neutral red. ×105.*

Effect of preliminary incubation at 37°C on virus and antibody titers. Serum-virus mixtures and virus-diluent mixtures were incubated for varying periods of time at 37°C prior to inoculation onto cell monolayers to determine the optimal incubation period for demonstration of CMV-neutralizing antibody. Table 2 shows the results of a representative experiment, and it is seen that virus titers were comparable after 30 and 90 min of incubation but were decreased by incubation at 37°C for 180 min. Neutralizing antibody titers were increased by longer incubation of the serum-virus mixtures at 37°C but, because of the inactivation of virus that occurred with 180 min of incubation, incubation for 90 min was adopted for routine use.

Enhancement of CMV-neutralizing antibody titers by fresh guinea pig complement. Comparative studies were performed to determine whether the enhancement of CMV-neutralizing antibody activity by complement reported for certain other plaque reduction neutralization systems (2, 6, 8) would also occur in the present system. Hyperimmune animal sera and sera from human CMV infections (diagnosed on the basis of a significant increase in CF antibody titer) were titrated in parallel in the presence of fresh guinea pig serum and heat-inactivated guinea pig serum. Table 3 shows that complement markedly enhanced CMV neutralization by hyperimmune animal sera, but a lesser degree of enhancement was seen for convalescent-phase sera from human CMV infections. The sensitivity of the CMV plaque reduction neutralization test incorporating complement into the reaction mixtures is further illustrated in Table 4, which shows that in the presence of complement, the test developed in this laboratory demonstrated slightly higher titers for monkey and goat hyperimmune sera to CMV strains AD-169 and C87 than were obtained by the Plummer and Benyesh-Melnick method (9) in the presence of

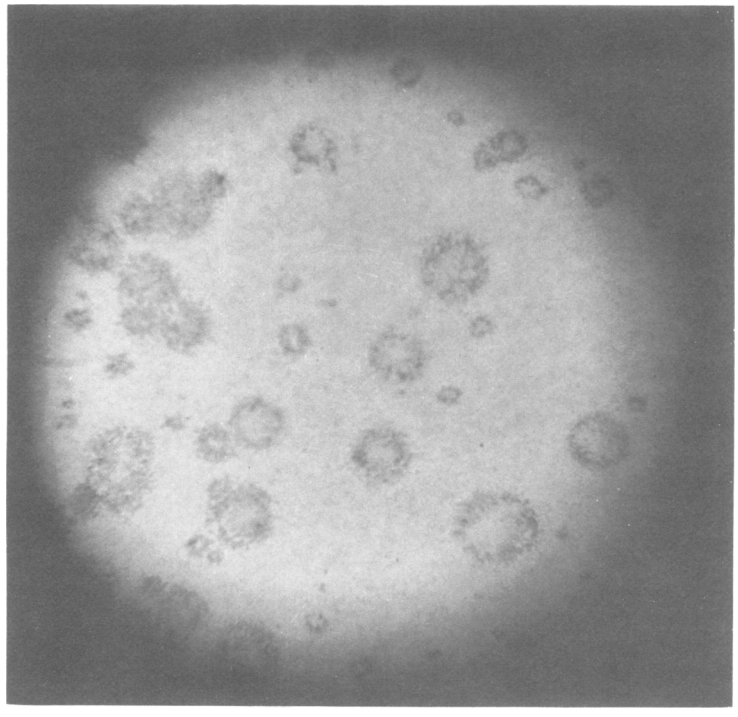

FIG. 2. *Foci of CMV-infected cells stained with neutral red 12 days after infection.* ×5.

TABLE 1. *Size and number of CMV plaques stained with neutral red at various times after infection*

Expt no.	Solidifying agent in overlay	Time of addition of neutral red overlay (day)	Plaque size (mm)	Virus titer (PFU/ml)[a]
1	Ionagar no. 2	6	0.2–0.3	6.0×16^6
		7	0.2–0.5	1.0×10^7
2	Ionagar no. 2	7	0.2–0.5	1.3×10^7
		11	0.8–1.5	1.0×10^7
3	Agarose	6	0.2–0.3	3.0×10^7
		7	0.2–0.5	5.0×10^7
		12	0.8–1.5	8.0×10^7

[a] Based on plaque counts in four or more wells.

complement. These sera were not available in sufficient quantities to permit testing in our system in the absence of complement.

As reported by others (2, 6), certain guinea pig sera were found to have heat-labile inhibitory activity for human CMV, and it was necessary to screen sera from individual animals for use in the plaque reduction neutralization test.

DISCUSSION

The modified plaque assay and plaque reduction neutralization test for human CMV described herein possess advantages over certain previously described assays (1, 9, 12) in that results may be obtained earlier and fewer ma-

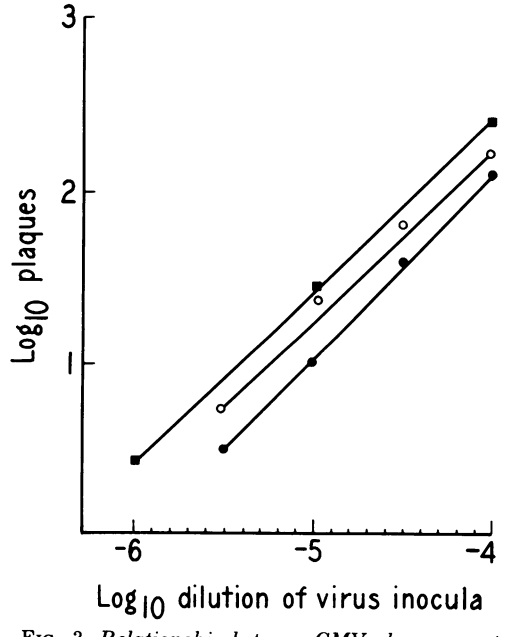

FIG. 3. *Relationship between CMV plaque counts and virus concentration. Results of three different titrations of CMV stock preparations.*

TABLE 2. *Effect of preliminary incubation at 37°C on virus and antibody titers*

Time of incubation of virus or serum-virus mixtures (min)	Avg no. of CMV plaques at $\log_{10}$ dilution:					CMV-neutralizing antibody titers of sera of patient:				
						K. Br.		C. Jo.		R. Li
	−4.0[a]	−4.5	−5.0	−5.5	−6.0	A[b]	C	A	C	C
30	112	39	14	4	1	<8	32	<4	16	8
90	117	41	12	3.5	1.5	8	32	<4	64	16
180	76	15	8	2	0.5	32	64	8	256	64

[a] Test dose of virus used in neutralization test.
[b] A, Acute-phase serum; C, convalescent-phase serum.

TABLE 3. *Effect of complement on CMV-neutralizing antibody titers of hyperimmune animal sera and convalescent-phase sera from human infections*

Serum tested	CMV-neutralizing antibody titer		CF titer
	−C[a]	+C	
Immune hamster			
Pre-immunization	<8	<8	<8
Early sera[b]	<16	64	ND[c]
Late sera	32	1,024	256
Human convalescent			
J. To.	64	512	128
B. Do.	1,024	2,048	1,024
R. Ro.	128	256	256
E. Se.	128	256	128
B. Mc.	128	512	256

[a] −C, Heated guinea pig serum used in test; +C, unheated guinea pig serum containing approximately 10 hemolytic units of complement used in test.
[b] Early sera = pooled sera collected 2 to 5 weeks after the beginning of immunization; late sera = pooled sera collected 6 and 7 weeks after the beginning of immunization.
[c] ND, Not done.

TABLE 4. *Neutralizing antibody titers of CMV reference antisera as determined in two different plaquing systems*

Antiserum (strain)	Host	Neutralizing antibody titer vs AD-169		
		Plummer-Benyesh-Melnick system (9)		Neutral red staining system (+C)
		−C[a]	+C	
AD-169	Monkey	<32	256	512
C87	Monkey	<32	256	2,048
C87	Goat	<32	2,048	8,192

[a] C, Complement absent; +C, complement present.

nipulations of the infected cell cultures are required. If the time of staining is delayed until 12 days, results can be read macroscopically in a time comparable to that required by other procedures for reading with low-power magnification.

The sensitive plaque reduction assay for CMV-neutralizing antibodies is expected to find use in antigenic analyses on human CMV strains and for more definitive studies on the sequential appearance of neutralizing and CF antibodies in human CMV infections and on the occurrence of CMV-neutralizing antibodies in various classes of immunoglobulins in initial and reactivated infections.

The production of high-titered, cell-free CMV for use in these studies was based upon the findings of other investigators that high yields of extracellular virus could be obtained in roller bottle cultures incubated for extended periods after the initial appearance of viral cytopathic effect (3, 5, 7) and that the late harvests of human CMV were more temperature stable than were early harvests (10). The ability to use challenge virus at relatively high dilutions should reduce the amount of noninfectious viral particles capable of binding antibody and, thus, should increase the sensitivity of CMV-neutralizing antibody assays.

Our results on enhancement of neutralizing antibody to the AD-169 strain of human CMV by fresh guinea pig serum, presumably due to complement, were in general agreement with those reported by others (2, 6, 8), namely, that antibody present in hyperimmune animal sera showed marked enhancement, whereas antibody in sera from human infections was enhanced to a lesser extent. However, the fact that titers of human sera were invariably enhanced by two- or fourfold indicates the desirability of routinely including complement in the test system, particularly to detect low levels of antibody. The importance of screening guinea pig sera for viral inhibitory activity prior to using them as a source of complement must be

stressed. It remains to be determined whether the heat-labile CMV inhibitory activity in the sera of certain animals represents a single, nonspecific substance or whether the activity is due to low levels of viral antibody that is complement dependent and becomes undetectable when the serum is heat inactivated. The fact that certain guinea pig sera which are inhibitory for CMV have no such activity for varicella-zoster virus suggests some level of specificity.

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LITERATURE CITED

1. **Anderson, H. K.** 1971. Cytomegalovirus neutralization by plaque reduction. Arch. Gesamte Virusforsch. 35:143-151.
2. **Anderson, H. K.** 1972. The influence of complement on cytomegalovirus neutralization by antibodies. Arch. Gesamte Virusforsch. 36:133-140.
3. **Chambers, R. W., J. A. Rose, A. S. Rabson, H. E. Bond, and W. T. Hall.** 1971. Propagation and purification of high-titer human cytomegalovirus. Appl. Microbiol. 22:914-918.
4. **Dennis, J.** 1975. A multi-purpose laboratory light box. Am. J. Med. Technol. 41:75-77.
5. **Furukawa, T., A. Fioretti, and S. Plotkin.** 1973. Growth characteristics of cytomegalovirus in human fibroblasts with demonstration of protein synthesis early in viral replication. J. Virol. 11:991-997.
6. **Graham, B. J., Y. Minamishima, G. R. Dreesman, H. G. Haines, and M. Benyesh-Melnick.** 1971. Complement-requiring neutralizing antibodies in hyperimmune sera to human cytomegaloviruses. J. Immunol. 107:1618-1630.
7. **Huang, E.-S., S.-T. Chen, and J. S. Pagano.** 1973. Human cytomegalovirus. I. Purification and characterization of viral DNA. J. Virol. 12:1473-1481.
8. **Minamishima, Y., B. J. Graham, and M. Benyesh-Melnick.** 1971. Neutralizing antibodies to cytomegalovirus in normal simian and human sera. Infect. Immun. 4:368-373.
9. **Plummer, G., and M. Benyesh-Melnick.** 1964. A plaque reduction neutralization test for human cytomegalovirus. Proc. Soc. Exp. Biol. Med. 117:145-150.
10. **Vonka, V., and M. Benyesh-Melnick.** 1966. Thermoinactivation of human cytomegalovirus. J. Bacteriol. 91:221-226.
11. **Waner, J. L., and J. E. Budnick.** 1973. Three-day assay for human cytomegalovirus applicable to serum neutralization tests. Appl. Microbiol. 25:37-39.
12. **Wentworth, B. B., and L. French.** 1970. Plaque assay of cytomegalovirus strains of human origin. Proc. Soc. Exp. Biol. Med. 135:253-258.