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Antisera to Human Cytomegalovirus Produced in Hamsters: Reactivity in Radioimmunoassay and Other Antibody Assay Systems

BAGHER FORGHANI, NATHALIE J. SCHMIDT, AND EDWIN H. LENNETTE*
Viral and Rickettsial Disease Laboratory, California State Department of Health, Berkeley, California 94704

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Hamsters immunized with human cytomegalovirus (CMV) concentrated and purified by polyethylene glycol precipitation and density gradient centrifugation produced antisera with high titers of specific viral antibody, and which showed no significant reactivity with human host cell components. The antisera had high titers of CMV antibody in complement fixation, indirect fluorescent-antibody (FA), and neutralization tests, but titers obtained by indirect radioimmunoassay (RIA) were markedly higher. The antisera were used to follow the development of CMV antigen in infected host cells by indirect RIA and indirect FA staining. Virus-specific antigen was first detectable by RIA at 8 h after infection, and by FA staining at 16 h; cells contained optimal amounts of antigen for RIA and FA assays at 72 to 100 h postinfection. Immune globulins from the antisera were labeled with ^{125}I for use in direct RIA. The labeled globulins gave highly specific reactions with CMV-infected cells, including those infected with low-passage isolates, and showed no reactivity with cells infected with other human herpesviruses or certain other human viruses.

The development of immunological methods for the specific identification of human cytomegalovirus (CMV) strains isolated in cell cultures, or for detection of viral antigen in clinical materials, has been severely hampered by inability to produce high-titered immune animal sera free from antibodies to human host cell components. Although antisera have been produced in a variety of species including rabbits (1, 19), guinea pigs (15), goats (10), baboons, and monkeys (9) by immunization with unpurified virus preparations, they have been satisfactory only in neutralization test systems, due to their high degree of reactivity with human host cells. Antisera prepared by immunization of rabbits with soluble CMV antigen extracted from the host cells with alkaline buffers (18, 23) have had less host cell reactivity, and have been used in complement fixation (CF) and fluorescent-antibody (FA) systems, but they have possessed little or no neutralizing antibody activity.

By immunizing guinea pigs with highly purified CMV, Huang et al. (12) were able to produce high-titered antisera free from antibodies to human host cell proteins which were suitable for use in CF and FA test systems. Using a minor modification of their methods for concentration and purification of immunizing antigen, we have produced high-titered CMV antisera in hamsters. The sera were free from host-cell antibodies and were suitable for use in a variety of immunological systems including radioimmunoassay (RIA), FA staining, CF tests, and neutralization tests. This report describes the preparation of the antisera, their reactivity in various antibody assay systems, and their specificity for identification of human CMV isolates by solid-phase direct RIA.

MATERIALS AND METHODS

Virus strains. The AD-169 strain of human CMV was used for immunization of the hamsters, and in the various antibody assay systems. Seed virus preparations with infectivity titers $\geq 6 \times 10^6$ plaque-forming units (PFU) per ml were prepared by subpassaging the virus four to five times at high multiplicities of infection. Ten CMV strains isolated in this laboratory from clinical specimens were also examined in these studies; they were at the third to fourth passage levels. A simian CMV strain recovered in this laboratory from uninoculated primary rhesus monkey kidney cell cultures, and identified as CMV by typical acridine orange staining of intranuclear inclusions, was also included.

Cell cultures. A strain of human fetal diploid lung (HFDL) cells established by J. H. Schieble of this laboratory was employed for most of the studies. The sensitivity of this strain to CMV was shown to be comparable to that of WI-38 cells obtained from

Flow Laboratories. Cells were routinely propagated on fortified (containing twice the standard concentrations of vitamins and amino acids) Eagle minimum essential medium supplemented with 10% fetal bovine serum. For maintenance of the cells during viral infection, the concentration of fetal bovine serum was reduced to 2%.

Preparation of immunizing antigen. HFDL cultures in roller bottles were infected with cell-free CMV at a multiplicity of ≥1 PFU per cell and incubated at 36°C (2). At 24 h after infection, over 90% of the cells showed the early, rounded cytopathic effect typical of cultures heavily infected with CMV (7). Cultures were incubated for an additional 7 to 8 days to obtain maximum yields of extracellular virus (7, 11). The cells were dislodged from the glass surface by shaking with glass beads, and the culture material was centrifuged at 5,000 rpm for 30 min. The supernatant fluid (1,000 ml) was collected, and polyethylene glycol 6000 was added to a concentration of 5%; a magnetic stirring bar was used to aid solution. After holding overnight at 4°C, the precipitate that formed was sedimented by centrifugation at 5,000 rpm for 30 min. The supernatant fluid was removed, and the precipitate was suspended in 10 ml of tris(hydroxymethyl)aminomethane (Tris)-buffered saline, pH 7.4 (0.05 M Tris-0.15 M NaCl) containing a 1% concentration of normal hamster serum (pooled preimmunization sera from the animals used for production of immune serum). After holding at 4°C for 4 to 6 h, the suspension was clarified by centrifugation at 2,000 rpm for 30 min, and the supernatant fluid was collected. This represented approximately a 100-fold concentrate of the original culture fluid.

Further purification of virus in the concentrate was achieved by the density gradient centrifugation procedures of Huang et al. (12). Five-milliliter volumes of the concentrate were centrifuged on a 25-ml gradient of 10 to 50% sucrose, prepared in Tris-buffered saline at 22,000 rpm for 2 h at 4°C. The bands that appeared at the 42 to 44% concentration of sucrose were collected and pooled. Preimmunization hamster serum was added to a concentration of 1%, and the virus was dialyzed against Tris-buffered saline, and then centrifuged on a 25-ml preformed cesium chloride gradient (density, 1.5 to 1.35), prepared in Tris-buffered saline, at 22,000 rpm for 2 h at 4°C. The light-scattering bands were collected from the gradient, and hamster serum was added as described above. The fractions were dialyzed against Tris-buffered saline, and then examined by electron microscopy and assayed for infectivity. The fractions showing the highest infectivity titers and greatest numbers of viral particles (see Table 1) were pooled for use as immunizing antigen.

Immunization of hamsters. Animals were screened for preexisting antibody to human CMV using the highly sensitive indirect solid-phase RIA procedure (5, 6). None of the hamsters showed antibody to human CMV. The purified virus preparation was mixed with an equal volume of Freund complete adjuvant, and the animals received 0.5 ml of inoculum by either the footpad or intraperitoneal (i.p.) route. Four injections were given at 2-week intervals, and the antibody response was monitored by trial bleedings at weekly intervals.

Radioimmunoassays. The indirect solid-phase RIA method used for assay of CMV antibodies was essentially the same as that described previously for detection of antibodies to herpes simplex virus (5, 6). CMV-infected HFDL cells in the bottom of 1-dram (ca. 3.7 ml) glass vials were used as the source of antigen. Cells suspended in growth medium were infected with cell-free CMV at a multiplicity of ≥1 PFU per cell, and incubated at 37°C for 1 h with occasional shaking; then the cell concentration was adjusted to 100,000 cells per ml and vials were seeded with 1 ml of the suspension. After incubation at 36°C in a CO_2 incubator for 24 h, the growth medium was replaced with maintenance medium, and incubation was continued for an additional 3 days, at which time the cells were fixed with acetone. Fluorescent-antibody staining showed that approximately 100% of the cells contained viral antigen. Uninfected HFDL cell cultures were prepared in glass vials in parallel by seeding with 50,000 cells. The number of counts per minute obtained with the test serum against CMV-infected cells was divided by the number of counts per minute of the serum against uninfected cells to obtain a binding ratio; ratios of 2.1 or greater were considered indicative of the presence of viral antibody in the test serum.

For use in the direct RIA system, the immunoglobulin G (IgG) fraction of the CMV immune sera was purified and labeled with ^{125}I by the chloramine T method (13, 20) exactly as described previously for purification and labeling of anti-species immune globulins (5, 6). The specific radioactivity of the labeled IgG was approximately 0.5 μCi/μg of protein. The labeled globulins were diluted in 0.01 M phosphate-buffered saline containing 5% fetal bovine serum to contain approximately 50,000 cpm in a volume of 0.1 ml. Binding ratios of 2.1 or greater, obtained by dividing the number of counts per minuted obtained with the labeled globulins against infected cells by the number of counts per minute obtained against uninfected cells, were considered indicative of the presence of CMV antigen in the test material.

Fluorescent-antibody staining. CMV-infected HFDL cells prepared as described above for RIA were planted in wells on glass slides at a concentration of 40,000 cells in a volume of 0.1 ml and incubated as described above. Indirect FA staining was done by the standard procedure of this laboratory (22). Antibody end points were based upon virus-specific nuclear staining of CMV-infected cells. For direct FA staining, the IgG fraction of the CMV immune serum was conjugated with fluorescein isothiocyanate by the method of Riggs et al. (21), and the conjugate was further purified and concentrated five times by ultrafiltration with a membrane cone (Amicon Corp., Lexington, Mass.).

CF tests. CF antibody assays were performed by the standard procedure of this laboratory (17) using antigens prepared both by freezing and thawing and by alkaline buffer extraction of CMV-infected cells (4).

Plaque assay and neutralization tests. A plaquing procedure for human CMV recently described from this laboratory (21a) was used for infectivity assays and plaque reduction neutralization tests.

RESULTS

Concentration and purification of human CMV. Concentration with polyethylene glycol had no adverse effect on CMV infectivity. The starting cell culture fluid had an infectivity titer of 10^6 PFU per ml, and the 100-fold concentrate had a titer of 10^7 PFU per ml, which is an overall loss in infectivity that would be expected with any virus concentration procedure. However, as shown in Table 1, centrifugation of the virus in a sucrose density gradient resulted in the loss of several logs of infectivity. Subsequent centrifugation in a CsCl gradient caused no additional loss of infectivity. Fraction 2 from the sucrose density gradient contained the highest concentration of infectious virus, nucleocapsids, enveloped virions, and dense bodies containing CMV-specific antigens (3), and this was further purified by centrifugation on the CsCl gradient. Fractions 3 and 4 from this gradient were pooled and stored at −70°C for use as immunizing antigen.

Development of CMV antibody in immunized hamsters. Indirect RIA and indirect FA staining were used to study the development of antibody to CMV, and to uninfected host cells, over the course of immunization of the hamsters. Figure 1 shows the RIA results on a representative animal immunized by the i.p. route. Sera collected before immunization and at weekly intervals thereafter were assayed at a 1:1,000 dilution against CMV-infected and uninfected cells. Increasing the number of immunizations was seen to increase the reactivity of the antisera with virus-infected cells. Reactivity with uninfected cells increased little until after the fourth immunization, at which time there was a sharp increase in host cell reactivity, which resulted in a lower binding ratio for infected to uninfected cells. All of the individual animals immunized by either route showed similar development of antibody to infected and uninfected cells by RIA, and titration of reactivity with virus-infected and uninfected cells by indirect FA staining also showed maximum specific viral antibody activity in week 5 and 6 bleedings, and then a sharp increase in reactivity with uninfected cells. Sera from week 5 and 6 bleedings of animals in each immunization group (footpad or i.p.) were pooled and used for further studies.

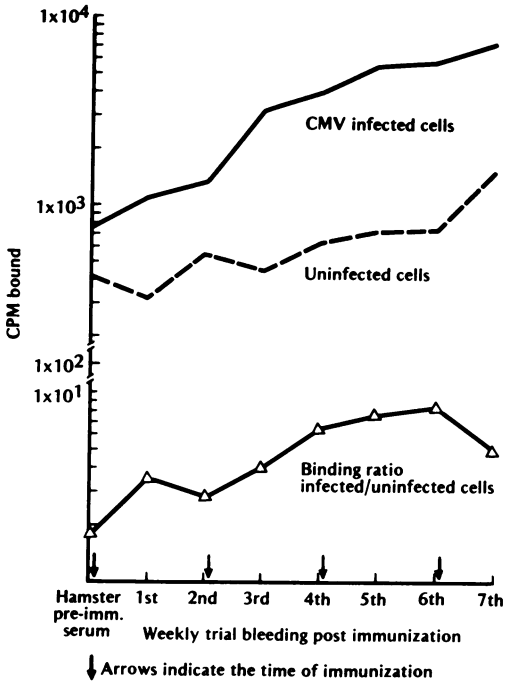

FIG. 1. *Development of CMV antibody in immunized animals.*

TABLE 1. *Recovery of CMV in density gradient fractions*

Fraction no[a]	Sucrose density gradient, 10–50%		CsCl gradient, density 1.15–1.35	
	Infectivity	Electron microscopy	Infectivity	Electron microscopy
1	None[b]	Amorphous particles	None	Amorphous particles
2	1×10^{4}[c]	Enveloped virions, nucleocapsids, dense bodies	None	Few nucleocapsids, rare dense body
3	1×10^{1}	Few enveloped virions, nucleocapsids, dense bodies	1×10^{2}	Nucleocapsids, dense bodies, rare enveloped virion
4	None	Amorphous particles	5×10^{3}	Enveloped virions, nucleocapsids, dense bodies
5	None	Amorphous particles	None	Amorphous particles

[a] Fraction numbers: 1, most dense; 5, least dense.
[b] No infectivity demonstrable in undiluted sample.
[c] Plaque-forming units per milliliter.

Titration of CMV immune hamster sera. The pooled antisera from each group of animals were titrated in various antibody assay systems. Figure 2 shows the results of indirect RIA titrations on immune and preimmunization sera from hamsters immunized by the i.p. route. The immune serum showed significant (≥2.1) binding ratios for infected to uninfected cells at dilutions through 1:256,000. Although the immune serum showed markedly higher specific binding ratios than the preimmunization serum, it is noteworthy that the latter showed greater reactivity with CMV-infected cells than with uninfected cells. This is likely due to the reactivity of the serum with the IgG receptors in the cytoplasm of CMV-infected cells, which have been demonstrated by others (8, 14) using FA staining.

Table 2 compares the titers in various assay systems of antisera from animals immunized by the two different routes. In each test system antisera from animals immunized by the footpad route had a titer twofold lower than that of animals immunized by the i.p. route, but they also showed slightly less host cell reactivity by CF. The antisera had high titers in each assay system, but RIA gave titers markedly higher than those obtained in conventional antibody assays.

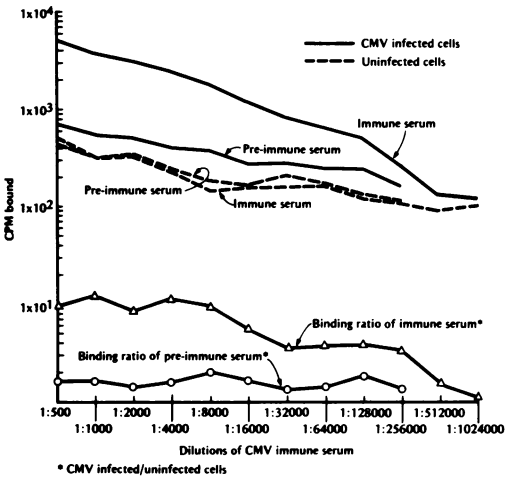

FIG. 2. *Titration of CMV immune serum by indirect RIA.*

Devleopment of CMV antigens demonstrable by RIA and FA staining of infected cells. Experiments were conducted to determine the earliest time at which virus-specific antigen was demonstrable, and the optimal time for demonstration of antigen in CMV-infected cells by RIA and FA staining.

Monolayers of HFDL cells were infected with extracellular CMV at a ratio of 1 PFU per cell and incubated at 36°C. At intervals over a 144-h period, representative cultures were fixed and examined by indirect RIA against a 1:1,000 dilution of preimmunization and CMV immune serum. In the RIA system, viral antigen was first detectable at 8 h postinfection. Optimal binding ratios with CMV immune serum were not obtained until 72 to 110 h postinfection, and by 120 h the release of antigen from the cells resulted in markedly reduced binding of the immune serum.

When the same sera were used in the indirect FA system to examine CMV-infected cells at various intervals after infection, viral antigen was first demonstrable at 16 h postinfection, and the weak staining reaction was confined to the nucleus of the infected cells. By 24 h there was a diffuse cytoplasmic staining and more intense nuclear staining. At 72 h nuclear inclusions and perinuclear staining were demonstrable, and cytoplasmic staining was more intense. Optimal staining was seen between 72 and 100 h postinfection; although there was cytoplasmic staining, the virus-specific nuclear staining was much more marked. At 120 h both cytoplasmic and nuclear staining were diminished, and by 160 h staining was lost completely. When the CMV antisera were conju-

TABLE 2. *Titers of CMV antisera in various serological test systems*

Route of immunization	Antibody titer by:			
	Complement fixation	Fluorescent-antibody staining	Plaque reduction neutralization	Radioimmunoassay
Footpad	2,048[a] (8)[b] 128[c] (<8)	2,048 (<16)	512	128,000 (<500)
Intraperitoneal	4,096[a] (16) 256[c] (8)	4,096 (<16)	1,024	256,000 (<500)

[a] Antigen produced by extraction with alkaline buffer.
[b] Titer against uninfected cells shown in parentheses.
[c] Antigen produced by freezing and thawing infected cells.

gated with fluorescein isothiocyanate and used in direct FA staining with CMV-infected cells fixed at 100 h postinfection, the nuclear staining was more intense, and the cytoplasmic staining was less marked than that seen by indirect FA staining. These findings are in agreement with those obtained by Laing (16) using human antisera for direct and indirect FA staining of CMV-infected cells.

Reactivity of CMV antisera in direct solid-phase RIA. Immune globulins purified from the CMV antisera and labeled with ^{125}I (6) were diluted as described under Materials and Methods and applied in a volume of 0.1 ml to CMV-infected and uninfected cells. Preliminary experiments showed that optimal binding of the labeled globulins to CMV-infected cells occurred with incubation times of 1 to 4 h at room temperature. After 4 h there was an increase in reactivity with uninfected cells, resulting in a lower specific binding ratio. An incubation time of 2 to 3 h at room temperature was adopted for direct RIA.

The specificity of the ^{125}I-labeled CMV immune globulins was evaluated by testing against cells infected with various herpesviruses and certain human viruses from other groups. The labeled globulins were also tested against 10 low-passage CMV isolates that had been recovered in HFDL cells. Cells from tube cultures of the isolates were dispersed with trypsin, suspended in 1 ml of maintenance medium, and inoculated in a volume of 0.1 ml into monolayer HFDL cultures in vials. After incubation at 36°C for 100 h, the cultures were examined by direct RIA.

Table 3 shows that the labeled CMV immune globulins reacted specifically with cells infected with the homologous AD-169 virus strain, and to a lesser extent with cells infected with a simian CMV. A positive reaction was also obtained with all 10 of the low-passage CMV isolates. No specific reactivity was seen with cells infected with other human herpesviruses, cells infected with measles, vaccinia, rubella, or mumps viruses, or any of the uninfected cells examined.

DISCUSSION

These studies confirmed the findings of Huang et al. (12) that high-titered CMV antisera free of antibodies to host cell components could be produced by immunization with purified virus derived from the fluid phase of infected cell cultures, and they demonstrated another laboratory host, the hamster, in which CMV antisera could be produced. Initial concentration of the virus by precipitation with polyethylene glycol as done in these studies might have some advantage over pelleting by high-speed centrifugation; it permits concentration of virus from large volumes of culture fluid using only short periods of low-speed centrifugation, and it caused no apparent loss in infectivity or antigenicity of the preparation. The concentrated virus was obtained in a small volume of fluid, and could then be purified by density gradient centrifugation without preliminary homogenization.

The investigations also defined an immunization schedule that produced immune hamster sera with maximum virus-specific activity and minimum host cell reactivity. Antisera from animals immunized by the footpad and i.p. routes differed little in either specific or nonspecific reactivity. The optimal time for harvest of CMV-infected cells for use in RIA and FA staining systems was also established.

TABLE 3. *Specificity of ^{125}I-labeled CMV immune globulins in direct solid-phase RIA*

Cells tested	cpm	Binding ratio, infected/uninfected cells
CMV-infected HFDL (AD-169 strain)	3,302	12.6
CMV-infected WI-38 (AD-169 strain)	3,667	14.3
Simian CMV-infected HFDL	1,622	6.2
Ten different CMV isolates in HFDL	624–1,864[a]	3.3–7.1[a]
Uninfected HFDL	261	
Uninfected WI-38	257	
Herpes simplex type 1 virus-infected HFDL	305	1.1
Herpes simplex type 2 virus-infected HFDL	246	1.0
Varicella-zoster virus-infected HFDL	320	1.2
Measles virus-infected HFDL	257	1.0
Vaccinia virus-infected HFDL	342	1.3
Rubella virus-infected BHK-21	248	1.0
Mumps virus-infected Vero	311	1.0
Uninfected BHK-21	350	
Uninfected Vero	315	

[a] Range of counts per minute or binding ratios.

Antisera to CMV produced in animals should give more definitive answers as to the degree of antigenic variation that exists among human CMV strains, and antisera produced in hamsters are suitable for use in several different assay systems, including the neutralization test, which is generally considered to be highly sensitive for detecting antigenic variation among virus strains.

Examination of the CMV antisera by RIA revealed several important findings. First, it demonstrated the markedly greater sensitivity of the indirect RIA test over that of CF, FA staining, and neutralization for detection of specific viral antibodies. The greater reactivity of preimmunization hamster serum with CMV-infected cells than with uninfected cells suggested that the IgG receptors demonstrable in the cytoplasm of CMV-infected cells by FA staining (8, 14) are also active in the solid-phase RIA system, which employs acetone-fixed virus-infected cells as a source of antigen. However, reactivity of immune sera with CMV-infected cells far exceeded that of the preimmunization sera.

The ^{125}I-labeled immune globulins from the CMV antisera gave a relatively strong reaction with a simian CMV, whereas little or no crossing between human and simian CMV strains has been demonstrable by neutralization (9, 10), CF, or FA staining (12). In fact, the same immune globulins conjugated with fluorescein gave only a weak reaction in FA staining with the simian CMV strain. This may reflect the greater sensitivity of RIA for detecting antigenic similarities between simian and human CMV strains, and the RIA procedure may prove valuable in better defining the degree of antigenic relatedness of these CMV strains.

A highly significant finding was the demonstration of the specificity of the ^{125}I-labeled CMV immune globulins only for cells infected with CMV. Not only did the labeled globulins fail to react with human viruses from heterologous groups, but they also showed no reactivity with cells infected with other human herpesviruses. The labeled globulins were highly sensitive for detection of the low-passage CMV isolates in infected cell cultures. This suggests that the direct RIA procedure with ^{125}I-labeled CMV immune globulins might be applicable to the rapid detection of CMV antigens directly in clinical materials such as urine sediments, blood leukocytes, or infected tissues.

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