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Indirect Hemagglutination Test for Detection of Antibodies to Cytomegalovirus

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An indirect hemagglutination test has been adapted for use with cytomegalovirus. The test is highly sensitive and reproducible. Both immunoglobulin M and immunoglobulin G antibodies can be detected by this method. The hemagglutination reaction can be inhibited by small amounts of homologous antigen. This principle permits early identification of virus isolated from diagnostic specimens.

The currently available methods for detecting antibody to cytomegalovirus (CMV) have several deficiencies. Some subclasses of immunoglobulin (Ig) G antibody do not fix complement (4), and IgM antibody is apparently nonreactive in the CMV complement fixation (CF) test (6). Although the neutralization test (8) is able to detect both IgM and IgG, it is a cumbersome method, and large scale testing is hampered by the slow growth of the virus. Reagents available for performing the indirect IgM fluorescent-antibody (FA) test (3) often produce inconsistent results, and the high quality fluorescein-conjugated anti-IgM serum required for the test is both expensive and difficult to obtain (10). To some extent, these deficiencies hinder research on the epidemiology and natural history of an infection widespread in human populations.

To overcome some of these problems, we have modified a previously described indirect hemagglutination (IHA) test (9, 12) for use with CMV. This paper discusses some of the results obtained with this CMV IHA test and compares them with the results obtained by complement fixation.

MATERIALS AND METHODS

Antigens. A standard CMV antigen was prepared from the AD-169 strain of virus. The same antigen was used for both CF and IHA. The *Herpesvirus hominis* type 2 antigen (V229) was prepared from a tissue culture isolate in our laboratory. The varicella-zoster antigen was obtained commercially (Microbiological Associates, lot 3-4122-GMK).

Tissue culture. Monolayers were prepared with the RU-1 strain of diploid human fetal lung fibroblasts. Eagle's medium as modified by Kissling and Reese (5) and supplemented with 10% fetal calf serum was used for propagation of cells; after growth, the cells were maintained in the modified medium supplemented with 2% fetal calf serum.

Antigen preparation. Tissue cultures propagated in 32-oz (ca. 960 ml) prescription bottles were inoculated with 5×10^5 to 10×10^5 $TCID_{50}$ of the AD-169 strain of CMV. When cytopathic effect (CPE), which consisted of enlarged, rounded, refractile cells, was observed in 80 to 90% of the culture, incubation was continued at 37 C for an additional 24 hr. Each infected monolayer was then rinsed with phosphate-buffered saline (PBS), and the cells were scraped into 2.5 to 5.0 ml of saline buffered at *p*H 9.0 with 0.05 M glycine-NaOH. The cell suspension was sonically treated for 5 sec at 25W with a Sonifier Cell Disruptor (model W185, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) with the microtip placed directly in the suspension. It was then clarified by centrifugation at $1,000 \times g$ for 10 min. The antigen was stored in samples at −60 C after the addition of dimethylsulfoxide to a final concentration of 10%. Antigens for IHA inhibition were prepared from tissue culture isolates in culture tubes by rinsing the tubes with PBS, scraping the cells into 0.5 ml of glycine buffer, and sonically treating as above.

Sera. Human sera with both positive and negative CF titers to CMV were tested. These came from the following groups: (i) infants suspected of having congenital infection with CMV, (ii) mothers of these infants, (iii) adults with post-transfusion mononucleosis or heterophile-negative infectious mononucleosis, and (iv) normal, adult blood donors and Center for Disease Control (CDC) personnel.

Human sera from patients with elevated titers and paired sera showing seroconversion to *H. hominis* and varicella-zoster were obtained from Bernard Lourié (Virology section, CDC). Sera from patients with heterophile-positive infectious mononucleosis and sera positive by the Epstein-Barr (EB) virus FA test were obtained from Paul Feorino (Virology section, CDC).

Serum fractions. Fractions containing IgM and IgG were obtained from selected sera by filtration through a Sephadex G-200 column, by using a 1.0-M NaCl solution, buffered at *p*H 8 with 0.0175 M PO_4

as an eluant. All sera were dialyzed against this buffer for 24 hr prior to fractionation. Serum samples of 0.5 to 2.0 ml were used for fractionation, and effluent fractions were collected in 5-ml volumes. These were tested for IgM and IgG content by double-diffusion precipitation in 1.5% agarose made in a 0.075 M barbital-NaOH buffer (*p*H 8) with commercial antisera to IgM and IgG (Behringwerke, certified Blood Donor Service, Woodbury, N.Y.). The tubes containing IgM were pooled, as were those containing the peak of IgG activity. The pooled fractions were dialyzed overnight against 0.15 M NaCl, buffered at *p*H 8 with 0.1 M glycine. They were then concentrated with carboxymethyl cellulose to a volume either two or four times the original serum volume. Because one passage through G-200 may result in some IgG in the void volume, the partially concentrated fractions were tested for contamination by the heterologous immunoglobulin by double-diffusion precipitation tests. No IgM was detected in any of the IgG fractions; however, small amounts of IgG were detected in some of the IgM fractions, producing very faint precipitation lines in the gel.

CF test. The CMV CF test was performed by the Laboratory Branch Complement Fixation microtiter method (2, 11).

IHA test. The IHA test was based, with minor modifications, on the procedures outlined by Stavitsky (12).

Phosphate-buffered saline. The buffered saline (*p*H 6.4) was prepared by mixing 100 ml of 0.15 M NaCl with 100 ml of a buffer composed of 32.2 ml of 0.15 M Na_2HPO_4 and 67.7 ml of 0.15 M KH_2PO_4. The buffered saline at *p*H 7.2 was prepared from solutions of Na_2HPO_4 and NaH_2PO_4 with a final concentration of 0.15 M NaCl and 0.01 M PO_4. Except where otherwise noted, the PBS of *p*H 7.2 was used in all procedures.

Diluent. The final suspensions of sensitized cells and all test serum dilutions were made in heat-inactivated normal rabbit serum which had been adsorbed with 50% sheep erythrocytes at 4 C for 30 min and diluted 1:100 in PBS.

Sheep erythrocytes. Citrated sheep blood 2 to 6 weeks old was washed three times with PBS. The packed cells were then diluted volumetrically to a 2.5% suspension and used the same day.

Preparation of tanned cells. Tannic acid (Baker reagent grade) was diluted to 1:20,000 just prior to use. Equal volumes of 2.5% erythrocyte suspension and the tannic acid solution were mixed and incubated in a 37 C water bath for 10 min. The cells were centrifuged at 500 × *g* for 10 min and washed once with PBS. They were then resuspended to 2.5% and used within 2 to 4 hr.

Sensitization of tanned cells. Four volumes of PBS (*p*H 6.4), 1 volume of the optimal dilution of sensitizing antigen, and 1 volume of 2.5% tanned erythrocytes were mixed in this order and kept at room temperature for 30 min. The cells were then centrifuged at 500 × *g* for 10 min, washed twice with rabbit serum diluent, and then adjusted to a 0.5% suspension in the same diluent.

All sera to be tested were diluted 1:10 in rabbit serum diluent, inactivated at 56 C for 30 min, and adsorbed at 4 C for 30 min by using 0.1 ml of 50% washed sheep erythrocytes per milliliter of diluted serum. Twofold dilutions in 0.05-ml volumes were then prepared in plastic microtiter U-plates. To each serum dilution, 0.05 ml of sensitized cells was added. The plates were sealed with clear tape, shaken, incubated at room temperature for 2 to 4 hr, and then refrigerated until read. Settling patterns were read as positive when the cells were completely and uniformly agglutinated or when a large circle of partially agglutinated cells coated the bottom of the well. Small rings of unagglutinated cells and buttons were read as plus-minus and negative, respectively. The highest serum dilution producing complete or partial agglutination was considered the end point. Controls consisted of (i) a 1:10 dilution of serum plus tanned erythrocytes treated with PBS, (ii) a 1:10 dilution of serum plus erythrocytes sensitized with uninfected RU-1 tissue culture antigen, and (iii) a positive control consisting of a complete titration of a known CMV-positive serum.

IHA-inhibition test. Equal volumes of a positive control serum and a suspension to be tested for CMV antigen were combined in a test tube and allowed to react at room temperature for 60 min. The absorbed serum was then used as indicated for the IHA test, and the test was read for specific inhibition of hemagglutination.

RESULTS

Antigen titration. The 1:8 dilution of CMV antigen was found to be optimal for sensitizing the tanned erythrocytes, both in terms of titer and in clarity of end point (Table 1). The RU-1 control antigen was likewise used at a 1:8 dilution.

Reproducibility. The positive control serum was titrated in every test with a reproducible end point of 1:160. Several sera were tested on multiple occasions and gave results reproducible within one twofold dilution.

In adapting the IHA test for use with CMV, we made no attempt to systematically vary each

TABLE 1. *Determination of antigen dilution for sensitization of tanned sheep cells*

Positive control serum dilution	Cytomegalovirus antigen dilution used in sensitization						
	Undiluted	1:2	1:4	1:8	1:12	1:24	1:32
1:10	+	+	+	+	+	+	+
1:20	+	+	+	+	+	+	+
1:40	+	+	+	+	+	+	+
1:80	+	+	+	+	+	+	+
1:160	±	±	±	+	+	+	±
1:320	−	−	−	±	±	±	−
1:640	−	−	−	−	−	−	−

of the test parameters to determine absolute optimal conditions. Some of the test conditions, however, were found to be critical for the proper performance of the test, and these will be discussed briefly.

Some variation was noted in the results obtained with different batches of sheep erythrocytes, primarily in the pattern produced by settled, nonagglutinated cells (and, therefore, in the sharpness of the end point) rather than in actual titer. Cells that were aged for at least 2 weeks gave generally sharper end points than fresh cells. Each new batch of cells was tested with the control serum prior to use. The formulation of the buffers was also found to be extremely important. The *p*H 7.2 buffer prepared by the method of Stavitsky (12) and having a final Na_2HPO_4-KH_2PO_4 buffer concentration of 0.075 M was found to be unsatisfactory in our test system since the cells were spontaneously agglutinated after the sensitization procedure. When this solution was replaced by a 0.01 M Na_2HPO_4–NaH_2PO_4 (*p*H 7.2) buffer, no further problems with spontaneous agglutination occurred.

The rabbit serum diluent was tested by using several serum concentrations. The best settling patterns were produced at dilutions of 1:100 or less. In the absence of serum the cells often failed to form compact buttons, and end points were difficult to read. Once the test conditions were established, the test was easily performed with excellent reproducibility.

Sensitivity. Sera from 200 "normal" adult blood donors and CDC personnel were tested by both IHA and CF for CMV antibodies. The comparative titers by the two methods are shown in Fig. 1. The IHA titers were generally 5 to 10 times higher than the CF titers but showed considerable scatter. All sera in this group found to be negative by CF (<1:8) were also negative by IHA (<1:10), and vice versa. The correlation coefficient for the two tests is 0.48 (omitting the <1:8, <1:10 in performing the calculation).

IHA inhibition. Incubation of a 1:40 dilution of the control serum (titer 1:160) with homologous and heterologous antigen preparations prior to adding sensitized erythrocytes gave the results shown in Table 2. Homologous antigen in dilutions as high as 1:16 produced complete inhibition of the hemagglutination reaction, whereas heterologous antigen (*H. hominis* type 2, varicella-zoster, RU-1 cells sonically treated) produced no inhibition, even at a 1:2 dilution.

The inhibition of the specific hemagglutination was extremely sensitive when the positive serum was used at a dilution near its end point (Table 3). Under these conditions, a 1:256 dilution of standard CF antigen completely inhibited the agglutination reaction, and a 1:1,024 dilution produced partial inhibition.

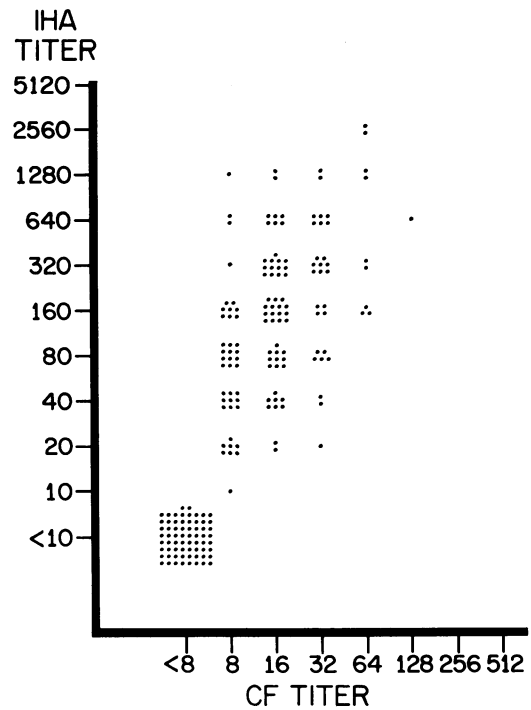

FIG. 1 *Comparison of IHA and CF titers in 200 normal individuals.*

TABLE 2. *Hemagglutination inhibition patterns produced by homologous and heterologous antigens*

Antigen used as inhibitor	Antigen dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Cytomegalovirus	−	−	−	−	±	±	+
Herpes type 2	+	+	+	+	+	+	+
Varicella-zoster	+	+	+	+	+	+	+
RU-1	+	+	+	+	+	+	+

Four antigens prepared from tissue culture isolates and identified as CMV by CF and FA were tested for IHA inhibition, and all four completely blocked the hemagglutination reaction. Another isolate which originally produced foci of enlarged cells resembling the CPE of CMV produced no IHA inhibition and was identified by CF and FA as *H. hominis*. IHA inhibition was used successfully to identify several suspected CMV isolates in single culture tubes showing moderate CPE 2 to 3 weeks after inoculation.

Cross-reactions. Paired sera showing either

TABLE 3. *Sensitivity of hemagglutination inhibition test with different serum dilutions*

Control serum dilution	Cytomegalovirus antigen dilution							
	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	No antigen
1:80	−	−	−	±	+	+	+	+
1:160	−	−	−	−	±	+	+	+
1:280	−	−	−	−	−	±	±	+
1:320	−	−	−	−	−	±	±	±

TABLE 4. *Serological cross-reactions between cytomegalovirus (CMV) and other herpesviruses*[a]

Serum	Herpes CF titer	V-Z CF titer	EBV FA titer	CMV CF titer	CMV IHA titer
1-S1	<4			<4	<10
S2	32			<4	<10
2-S1	16			<4	<10
S2	512			<4	<10
3-S1		8		16	160
S2		256		16	160
4-S1		<8		<4	<10
S2		16		<4	<10
5-S1			10	<8	<10
S2			50	<8	<10
6-S1			<10	<8	<10
S2			50	<8	<10
7-S1	16	<8		4	160
S2	128	>256		8	640

[a] Abbreviations: CF, complement fixation; V-Z, varicella-zoster; EBV, Epstein-Barr virus; FA, fluorescent antibody; IHA, indirect hemagglutination.

titer rises or seroconversion to *H. hominis* (CF), varicella-zoster (CF), and EB virus (FA) were tested for CMV antibodies by CF and IHA. Results are shown in Table 4. A serological rise to any of these agents in an initially CMV-negative individual produced no rise in titer to CMV by either CF or IHA. In one individual with a seroconversion to varicella and a pre-existing CMV titer, a boost in titer to CMV was found. In other similar cases, such a boost did not occur.

Infant sera. Table 5 shows the IHA titers in successive sera from infants with declining CF titers, which were thought to indicate passively acquired maternal antibody. The IHA titers

TABLE 5. *Duration of passively acquired antibody titers to cytomegalovirus*[a]

Infant serum no.	Age	IHA titer	CF titer	Maternal titer at time of infant S1	
				IHA	CF
A S1	3 days		64	160	128
S2	3 weeks	80	32		
S3	2 months	40	8		
S4	6 months	<10	<4		
B S1	10 days	320	32	640	16
S2	3 months	20	<4		
C S1	1 month	40	4		
S2	4 months	10	<4		
D S1	2 months	80	8		
S2	5 months	<10	<4		

[a] Abbreviations: IHA, indirect hemagglutination; CF, complement fixation.

followed a pattern similar to the CF but in two cases were still weakly positive at a time when CF antibody could no longer be detected (in infants less than 6 months old). In a group of 12 infants 6 months of age and older, selected on the basis of negative CF titers, no antibody was demonstrable by IHA.

In a series of 14 infants with congenital infection, antibody titers persisted beyond 6 months of age as determined by both CF and IHA. The titers of two of these infants are shown in Table 6.

Serum fractions. Sera from seven CMV mononucleosis patients and one normal blood donor (no. 5, Table 7) were separated into IgM and IgG fractions by Sephadex G-200 filtration and tested by CF and IHA (Table 7). Of the eight IgM fractions tested, five showed no evidence of IgG contamination as detected by immunodiffusion, whereas three fractions (no. 5, 6, and 8) contained traces of IgG. Hemagglutinating antibody was found in both fractions, but the distribution of antibody activity between the two fractions was variable. Significant CF antibody titers were found in most of the IgG fractions, whereas CF antibody was found in only one of the IgM fractions. Serum no. 5 had a very high IHA titer (1:1,280) which was entirely localized to the IgG fraction, and yet had a CF titer of only 1:8. The IgM fraction, despite contamination with IgG, showed no IHA activity.

DISCUSSION

Our IHA test for CMV is a modification of a widely used serological technique. Although the

TABLE 6. *Cytomegalovirus antibody titers in congenitally infected infants*[a]

Infant	IHA titer			CF titer		
	S1	S2	S3	S1	S2	S3
1	2,560 (newborn)	80 (4 months)	1,280 (7 months)	128	16	16
2	80 (3 months)	20 (9 months)		8	4	

[a] Abbreviations: IHA, indirect hemagglutination; CF, complement fixation.

TABLE 7. *Distribution of cytomegalovirus antibody activity in IgM and IgG serum fractions from adults with CMV antibody*[a]

Serum no.	Whole serum titer		IgM fraction titer		IgG fraction titer		IgG detected in IgM fraction by immuno-diffusion
	IHA	CF	IHA	CF	IHA	CF	
1	2,560	640	10	<10	640	160	No
2	1,280	160	20	<10	320	40	No
3	5,120	640	20	<20	1,280	320	No
4	2,560	4	1,280	<8	80	<8	No
5	1,280	8	<10	<8	1,280	<16	Moderate
6	10,240	512	5,120	<20	640	320	Trace
7	160	512	10	<10	80	128	No
8	320	320	160	8	320	160	Trace

[a] Abbreviations: IHA, indirect hemagglutination; CF, complement fixation; Ig, immunoglobulin.

IHA test results show a correlation with the CF results in a normal population, the correlation is not complete and significant exceptions can be seen, particularly in sera from individuals with clinical illness. Some sera have high IHA titers predominantly in the IgM fraction with low or negative CF titers, these results confirming the previously demonstrated insensitivity of the CMV CF test to IgM antibody (6). Such sera are representative of a group that would be expected to have high IHA and low CF titers in the early stages of infection when IgM predominates.

In another serum with an IHA titer disproportionately higher than the CF, all of the antibody activity was found in the IgG fraction. These results might be produced by sera containing an IgG antibody subclass that does not fix complement (4, 7) or by the presence in the IgG fraction of other specific immunoglobulins that fail to fix complement.

Several sera have been found to have disproportionately low IHA titers compared with the CF results. Most of these have been from patients with recent CMV infection. This group may have "early" antibody with different antigen-binding properties than those of the normal subjects who were presumably infected some years ago. Alternately, these findings may reflect differences in the antigens reacting in the two test systems.

We are investigating preliminary evidence which suggests that the CMV antigen sensitizing the sheep erythrocyte is a soluble antigen, whereas the antigen reacting in the CF test is a combination of both viral and soluble components (1). Different sera should contain different proportions of antibody to the viral and soluble antigens. Those sera containing antibody to soluble antigens should react equally well in either test; those containing antibody to only viral antigens should have disproportionately low IHA titers.

Although the IHA test generally gave serum titers from 5 to 10 times higher than the CF test (Fig. 1), few sera have been positive by IHA and negative by CF. It appears that these two tests are essentially equivalent with respect to detection of cases. This may be a reflection of the potency of our CF antigen; an antigen of lower potency might fail to detect antibody by CF at levels that are still detectable by IHA.

The specificity of the IHA test is demonstrated by the IHA-inhibition tests and by the absence of CMV seroconversion accompanying titer rises to related herpesviruses. In one case previously noted, a boost in a pre-existing CMV titer was concomitant with a varicella-zoster seroconversion, but in this patient boosts in titer to *H. hominis* and vaccinia also occurred. The antigenic relationships among these viruses are at present unclear.

The apparent specificity of the IHA test suggests its use to detect CMV strain differences. Virus isolates could be used either as sensitizing antigens or as inhibiting antigens in the IHA-inhibition test. The sensitivity of the IHA-inhibition test has allowed us to identify (on an immunological basis) virus isolates from single inoculated tubes several weeks before identification could be made by CF methods. These results suggest that the IHA-inhibition test might be adapted to identify CMV directly from infected urine.

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ADDENDUM IN PROOF

Since this manuscript was submitted, a similar microhemagglutination method for cytomegalovirus has been reported (D. A. Fuccillo et al., Appl. Microbiol. **21**:104–107, 1971). This method was previously abstracted (Bacteriol. Proc., p. 185, 1970).

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