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Analysis of Antibody Assay Methods and Classes of Viral Antibodies in Serodiagnosis of Cytomegalovirus Infection

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Forty-nine serum pairs with antibody to cytomegalovirus (CMV) were evaluated for rises in antibody titer (≥ fourfold) by indirect hemagglutination (IHA) and complement fixation (CF), using a freeze-thaw antigen (FT) and a glycine extract antigen (GE). In this sample CF-FT detected more rises in antibody titer than did CF-GE. IHA detected the least number. The apparent reason for stationary antibody titers with CF-GE and IHA was the presence of high antibody titers in the first serum specimen. Separation of immunoglobulin classes of 20 serum pairs by sucrose gradient centrifugation indicated that these antibodies with IHA were of the immunoglobulin M (IgM) class and those with CF-GE were of the IgG class. By separation of immunoglobulin classes, rises in IgG CMV antibody titers were seen with IHA, rises not observed in the whole serum because of high IgM antibody titers in the first serum specimen. Absence of rises in antibody titers with CF-FT was due in part to too early sampling of the second serum specimen (<21 days) and in part to an apparent inability of some individuals to respond with antibody reactive with FT antigen. CF-GE and CF-FT antibodies of the IgM class were detected in some sera, usually in specimens collected more than 10 days after the onset of symptoms. Although reactive with CMV antigen, the specificity of these IgM antibodies in relation to rheumatoid factor requires clarification.

The indirect hemagglutination test (IHA) is reported to be sensitive in the detection of antibody to cytomegalovirus (CMV) (5, 10, 14). The present study was initiated to determine the ability of IHA, as compared to complement fixation (CF), to detect rises in antibody titer for the serodiagnosis of CMV infection and to determine the classes of antibodies involved. Two CF antigens previously reported were used (9). One is a glycine extract (GE) of infected cells that consists mainly of nucleocapsids, dense bodies, and a few enveloped virions, and the other is a freeze-thaw antigen (FT) composed mainly of amorphous material with some enveloped virions and dense bodies and only a few nucleocapsids.

MATERIALS AND METHODS

Antigens. Antigens were extracted from human fetal diploid cultures infected with CMV (strain AD169) and from noninfected cells of the same line by 0.1 M glycine buffer, pH 9.5, or by three cycles of freezing and thawing of the cells as previously described (9).

Determination of antibody class. A 1:2 or 1:4 dilution of serum was centrifuged in a 10 to 40% linear sucrose gradient as previously described (9). Fractions (0.5 ml) were collected manually from the bottom of the tube. Each fraction was tested for immunoglobulin class by the Ouchterlony test, using antisera to human μ, γ, and α heavy chains (Behring Diagnostics, Somerville, N.J.). Selected gradients were tested by radial immunodiffusion, using plates specific for human heavy chains (Oxford Laboratories, Foster City, Calif.). The fractions were tested by CF with both GE and FT antigens and by IHA. For removal of anticomplementary effects the gradient fractions were treated with an equal volume of complement (guinea pig serum diluted 1:4 in Veronal-buffered saline). After incubation of the mixtures at 37°C for 30 min, they were incubated at 56°C for 30 min to inactivate the added complement.

Antibody assays. CF tests were done by a standard method (21), utilizing 2 U of each antigen as determined by block titration with the same standardized human serum pool. IHA tests were done according to the method of Bernstein and Stewart (5), with the following modifications. A 1:80,000 dilution of a 1% tannic acid solution was found to be optimal for treatment of sheep erythrocytes. The FT rather than the GE antigen proved to be better for coating the sheep erythrocytes. Agglutination patterns with GE-antigen-coated cells tended to be incomplete and did not give a definitive end point. Complete or almost complete agglutination by antibody of cells coated with the optimal dilution of FT antigen was taken as the end point. The concentration of antigen for use was determined by block titration with a positive reference

human serum. For reproducibility of the assay it was found necessary to use as reference a serum containing immunoglobulin M (IgM) antibody. Sheep erythrocytes were coated similarly with an FT extract of noninfected cells for use as a nonspecific control. A positive serum of known titer and a negative serum were included in each run.

Serum samples. Forty-nine paired sera submitted to our diagnostic laboratory for serological diagnosis of viral infection were tested for CMV antibody by CF with two CMV antigens and by IHA. The sera were from patients with clinical signs compatible with CMV infection; 10 of the 49 patients had, in addition, organ transplants, and five had recently undergone heart surgery. Forty-six of the 49 paired sera were from adults whose average age was 34.5 years, with a range of 19 to 77 years. Three paired sera were from children of 1, 4, and 12 years of age, none of whom had been congenitally infected with CMV. Twenty-three paired sera were selected for further study of the immunoglobulin class of CMV antibodies. Selection was made on the basis of a fourfold or greater rise in antibody titer between the serum pairs by one or more of the tests and by presence of antibody in the first specimen by one or more of the tests.

RF. Sera were checked for rheumatoid factor (RF) by using Hyland Laboratories' latex globulin reagent in the slide test. Positive specimens were further evaluated by the tube agglutination method, according to the directions with the Hyland Laboratories product.

RESULTS

Evaluation of procedures for rise in antibody titer. A fourfold or greater rise in antibody titer is considered evidence for current infection with the virus under test. The 49 paired sera selected for study had a fourfold or greater rise by one or more of the three tests.

An evaluation of the antibody response with time of collection of both the first and second serum samples after onset of symptoms showed that a collection date for the first serum of <10 days and one for the second serum sample of greater than 21 days increased the ability of FT antigen to detect rises, whereas with GE antigen the increase was not as marked. A rise in antibody titers of all of 10 serum pairs in this category was detected by CF-FT, and a rise in 9 of the 10 serum pairs was detected by CF-GE (Table 1). The ability of CF-GE to demonstrate significant antibody titer rises between serum pairs was greatly reduced with a collection date of the first serum sample beyond 10 days, whereas that of CF-FT was less affected (Table 1). The IHA test detected the least number of antibody titer rises regardless of the time of serum sampling.

Table 2 compares the CF and IHA geometric mean (GM) antibody titers of all of the first serum samples that were collected less than 10 days after the onset of symptoms and correlates them with the presence or absence of a fourfold or greater titer rise in the paired second serum samples. When no rise in antibody titer in the paired second serum sample was detected by CF-GE and IHA, the antibody titer in the first sample of the serum pair was already very high. The GM titer in such cases by CF-GE was 1:776, and by IHA it was 1:512. The reason for the lack of antibody titer rise by CF-FT was not the same as with CF-GE and IHA since the GM titer of the first serum samples was the same whether or not a titer rise occurred in the second sample (GM titers of 1:8 and 1:9, respectively).

TABLE 1. *Evaluation of assays for rise in antibody titers*

First/second time of serum collection (days)[a]	No. of patients	CF		IHA
		GE	FT	
≤10/≤21	18	83.4[b]	77.8	66.7
≤10/>21	10	90.0	100.0	60.0
>10/>21	21	66.7	90.5	71.5
Average		77.6	87.8	67.4

[a] Time of serum collection after onset of symptoms.
[b] Percentage of serum pairs showing a rise in titer (≥fourfold).

TABLE 2. *GM titer of the first serum samples[a] in relation to a rise in titer between the first and second serum samples*

Test	GM titer of first serum samples	
	With rise in antibody titer in second sample	With no rise in antibody titer in second sample
CF-GE	28 ± 5[b]	776 ± 3
CF-FT	8 ± 2	9 ± 3
IHA	47 ± 6	512 ± 5[c]

[a] Sera collected <10 days from onset of symptoms; 18 serum pairs.
[b] Reciprocal of the GM titer with standard deviation.
[c] One serum pair in this group (not included in the calculation) showed no antibody response by IHA. If this were included, the GM titer would become 315 ± 8.

Immunoglobulin class of antibody. By density gradient centrifugation there was good separation of IgM and IgG antibodies in 20 of the 23 serum pairs. Where both IgM and IgG antibodies were present, a bimodal distribution of antibodies was seen (Fig. 1). IgM was present in fractions of the first peak of antibody activity, and IgG and IgA were present in fractions of the second peak. Fractions intermediate between the two peaks contained all three immunoglobulins. In 3 of the 23 serum pairs separation between IgM and IgG antibodies was not as

distinct as that shown in Fig. 1. Therefore, the data from these gradients are not included. The absence of a bimodal distribution of IHA antibody in these sera (Fig. 2) was attributable to the serum and not to the preparation of the gradient, since on repetition the same distribution occurred. The shape of the IHA curve suggested the presence of a high concentration of antibody of the IgA class. This was verified by indirect immunofluorescence, using specific anti-α-chain antibody labeled with fluorescein.

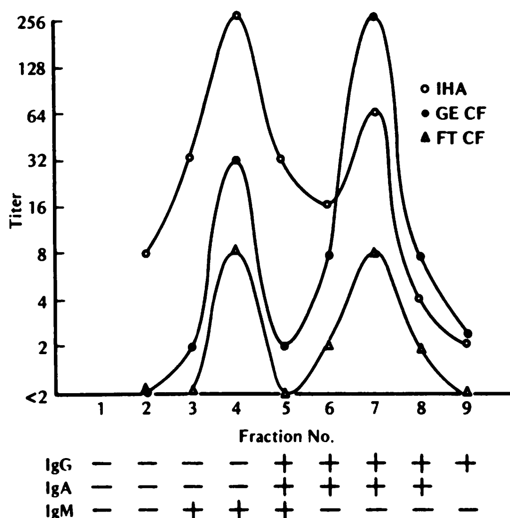

FIG. 1. *Sucrose gradient of a serum collected 30 days from onset of symptoms. Assay for RF was negative. Immunoglobulin data are from Ouchterlony analyses.*

Tables 3, 4, and 5 summarize the data on the 20 serum pairs that were satisfactorily studied for IgM and IgG classes of antibody. Table 3 shows that CF-GE antibody in IgM fractions does not appear in all sera and when it does it is usually delayed, whereas IHA IgM antibody appears earlier and in the majority of sera. Not all gradients were checked by CF-FT. Of those checked (gradients of 13 serum pairs), antibody activity was present in the IgM fractions of 5 sera taken longer than 10 days from onset of symptoms. The titers were two- to eightfold lower than those with GE antigen. Table 4 gives the GM titers of the IgM and IgG fractions. The CF titers for IgM antibody are low because of the many sera lacking antibody in the IgM frac-

TABLE 3. *Relationship between time of collection of serum and antibody class*

Time of serum collection (days)	Class of antibody	CF-GE	IHA
≤10	IgM	2/16[a]	11/16
≥11[b]	IgM	13/24	22/24
≤10	IgG	10/16[c]	7/16
≥11[b]	IgG	22/24	20/24

[a] Number of sera with IgM antibody/total number of sera.

[b] Times may be for either of the samples of the serum pairs.

[c] Number of sera with IgG antibody/total number of sera.

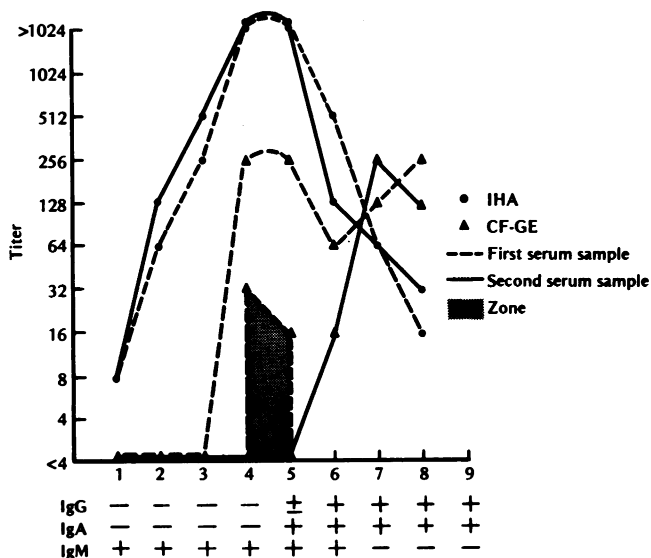

FIG. 2. *Sucrose gradient of a serum pair. Collection dates of the serum samples were 19 and 32 days. Shaded area indicates fractions with a prozone in the CF test.*

TABLE 4. *GM antibody titers to CMV by antibody class*

Serum sample	CF		IHA[a]
	GE[a]	FT[b]	
IgM antibody			
First	1:4 ± 5[c]	1:1	1:37 ± 8
Second	1:13 ± 8	1:7 ± 7	1:97 ± 8
IgG antibody			
First	1:20 ± 9	1:3 ± 2	1:8 ± 6
Second	1:231 ± 5	1:14 ± 3	1:104 ± 5

[a] Data from 20 serum pairs.
[b] Data from 13 serum pairs.
[c] Titer of fraction of the IgM or IgG class with the highest titer was taken as the titer of that particular antibody class. Titers of <1:4 were scored as 1:2 for purposes of determining the GM.

TABLE 5. *Percentage of serum pairs with rise in antibody titer of different classes*

Specimen	CF		IHA[a]
	GE[a]	FT[b]	
IgM fraction	35	30.7	35
IgG fraction	70	61.5	80
IgM + IgG fractions	80	76.9	80
Whole serum	70	84.6	65

[a] Number of serum pairs = 20.
[b] Number of serum pairs = 13.

tion. For calculation of GM titers, such sera were scored as 1:2. If the GM titer is calculated only on those sera having IgM antibody titers, the GM titer in the second serum sample by CF-GE is 1:60, and by IHA it is 1:194. The reverse occurs for IgG antibody. IgG antibody detected by CF-GE tends to arise earlier and is at a higher titer in first and second serum samples as compared with IgG antibody detected by IHA (Tables 3 and 4). By separation of the classes of antibody, fourfold or greater rises in antibody titers of different classes in serum pairs become apparent, rises that were not seen in the whole serum (Table 5).

The serum pairs studied by gradient centrifugation were evaluated for RF. Four sera, two first serum samples and two second serum samples, could not be checked because the amounts were inadequate. Eleven of 18 first serum samples and 10 of 18 second serum samples were negative for RF by the slide test. Only two serum pairs reacted strongly, one by tube test only and the other by slide and tube tests. The other positive sera were weakly positive on slide test and on tube agglutination demonstrated weak reactions, 1+ at a 1:40 or 1:80 dilution of serum. IgM CF and IHA antibodies were found in both RF-positive and RF-negative sera (Fig. 3). Two of three serum pairs not giving a bimodal distribution of antibody classes on gradient centrifugation were also RF positive.

DISCUSSION

It was not possible to determine if the infections in the present study were primary infections, second infections with the same or another strain of CMV, or reactivation of latent infections. In the latter two cases an anamnestic rather than a primary antibody response would occur, and antibody would appear earlier after infection. CMV infection is common, and the prevalence of CMV antibody in adults over 35 years of age is 51 to 81% (4). A check of the first serum samples with a titer of <1:8 showed that 45% of sera had <1:8 by CF-FT, 20% by CF-GE, and 12% by IHA. However, a titer of <1:8 in the first serum does not necessarily mean that the infection is a primary one, since the antibody titer from an infection in the distant past may have dropped below detectable levels. IgM antibody production is also not diagnostic for a primary infection but may occur as well upon reinfection (24, 25).

The 49 serum pairs examined in this study can be divided into two groups: one collected from 34 patients with symptoms of CMV infection only, and the other collected from 15 patients who, in addition to CMV infection, had transplants (10 patients) or heart surgery (5 patients). When the antibody response in serum pairs (the first sample of which was taken <10 days from onset of symptoms, and the second, <21 days) was separately tabulated for the two groups, the pattern of response was essentially the same for both (see Table 1). There were too few serum pairs taken at the other collection times (Table 1) from the group of heart surgery and transplant patients to evaluate this group separately.

The ability of the various procedures to detect antibody titer rises in the present sample of serum pairs was CF-FT > CF-GE > IHA (Table 1), whereas the sensitivity of the tests in detecting antibody was in reverse order, IHA > CF-GE > CF-FT. Failure of CF-GE and IHA to detect rises in antibody titer in these selected serum pairs was due to too high antibody titers in the first serum sample (Table 2). The negative findings with CF-FT could be correlated with too early sampling of the convalescent-phase serum or with an apparent inability by some patients as late as a month or more after onset of symptoms to produce antibody reactive with FT antigen. In our studies (9) approximately 5 to 10% of sera are negative or show low titers, i.e., <1:8 or 1:8 to 1:16. Other investigators have

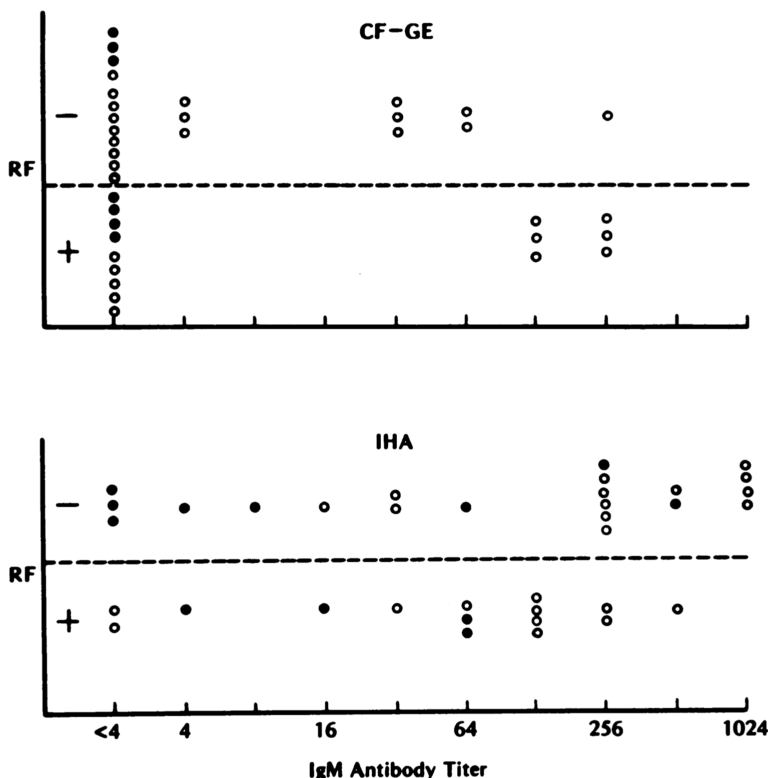

FIG. 3. *Relationship between presence or absence of RF by the latex globulin test and presence of IgM and IgG CMV antibodies by IHA and by CF with GE antigen. Both first and second serum samples are plotted. Closed circles indicate samples with no IgG antibody but which may or may not have IgM antibody as indicated by the antibody titers. Open circles indicate samples with IgG antibody, which may or may not have IgM antibody as indicated by the antibody titers.*

also reported the absence of immune response by some individuals to antigenic preparations made by simple mechanical disruption of infected cultures, either by cycles of freezing and thawing (1, 7) or by treatment in a VirTis blender (17). If only CF-FT is used in serodiagnosis, therefore, a significant percentage of patients will be misdiagnosed as negative for CMV. On the other hand, a rise in titer will be missed in over 20% of serum pairs by CF-GE if the collection of the first serum sample is delayed; however, in such cases the titer is very high, and one would suspect a current infection, which could possibly be verified by CF-FT.

Although the FT antigen was used in both the CF-FT test and the IHA test, the antibody response detected by the two tests was dissimilar. IgM antibody was detected more frequently by IHA and the response arose more quickly. This may reflect the greater sensitivity of IHA for IgM antibody or that different antigens are involved in the two tests. Thirty-two polypeptides have been isolated from CMV (11). Which of these polypeptides are in the FT antigen and which are involved in the reactions reported in this paper are not known.

In the early sera, the CF antibody is primarily IgG, whereas the IHA antibody is mainly IgM. The IHA test, although relatively inefficient in detecting rises in antibody titer in whole serum, was of the same or better efficiency as the CF test if only IgG antibody was measured (Table 5). The high IHA titers of IgM antibody in some first serum samples masked the rise in IgG antibody. The IgM antibody detected by CF arose later in the immune response than did the IHA IgM antibody (Table 3), which suggests that the reactive IgM antibody molecule is not the same in the two tests. The appearance of antibody classes in serum after antigenic stimulation is usually either IgM followed by IgG or the simultaneous appearance in the serum of the two classes, a pattern shown by IHA but not by CF in the present study. Langenhuysen et al. (20) and The and Langenhuysen (33) also reported the absence of IgM CMV antibody by the indirect fluorescent-antibody test until 11 days or more after development of symptoms.

There have been suggestions in the literature of two subclasses of IgM (22, 23), but this has not as yet been proven by immunochemical analyses.

Although there are a few reports of CF by IgM antibody with viral antigens (29, 34), most studies indicate an inability of IgM antibody to fix complement with either DNA viral antigens (12, 13, 18, 19, 27, 32) or RNA viral antigens (3, 6, 8, 15, 28, 30). The report of Shirodaria et al. (31) on false positive IgM reactivity by IFA in the presence of IgM RF-IgG viral antibody complexes has pertinence for the present study. Such complexes would band in the more dense fractions on sucrose gradient centrifugation similarly to specific viral IgM antibody. Although some sera were negative for RF and positive for viral IgM antibody by IHA and CF (Fig. 3), such data do not preclude the phenomenon, since the RF may be present in a concentration too low for detection by the latex globulin test. Similarly, lack of reaction for IgG in the Ouchterlony test or by radial immunodiffusion does not rule out its presence, since both RF and anti-γ-chain antibody react with the Fc portion of the IgG molecule. RF may sterically inhibit reaction of anti-γ-chain antibody with the IgG in the complex.

The presence of RF and IgG-specific antibody in a serum sample, however, does not necessarily mean that CMV antibody activity by CF will be present in IgM fractions, since five RF-positive sera with IgG antibody titers ranging from 1:64 to 1:8,192 did not have reactivity in the IgM-containing fractions (Fig. 3).

More definitive data may be obtained by absorption of the IgM fractions with aggregated IgG, and such tests are planned in extension of the present study.

Aberrant immunological responses that include antinuclear antibody, RF, cold agglutinins, and cryoglobulins may occur in CMV infection (2, 16, 26, 31, 35). RF has been reported also to arise in other viral diseases such as rubella, influenza, and herpes zoster (2, 30). Thus, the need for more discriminatory measures for identification of viral IgM antibody than are usually done is becoming more apparent for virus infections in general.

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