JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1984, p. 147–152
0095-1137/84/020147-06$02.00/0

Vol. 19, No. 2

Immunity to Human Cytomegalovirus Measured and Compared by Complement Fixation, Indirect Fluorescent-Antibody, Indirect Hemagglutination, and Enzyme-Linked Immunosorbent Assays

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Received 21 June 1983/Accepted 24 October 1983

The complement fixation test is currently the test employed most frequently to determine the presence of antibody to human cytomegalovirus. Several other techniques have been adapted for this purpose. A comparison of cytomegalovirus antibody titers was made between the complement fixation test, a commercially available enzyme-linked immunosorbent assay, an indirect immunofluorescent technique, and a modified indirect hemagglutination test. Forty-three serum samples were tested for antibodies by each of the above procedures. The enzyme-linked immunosorbent, immunofluorescent, and indirect hemagglutination assays were in close agreement on all samples tested; the titers obtained with these methods were all equal to or greater than the complement fixation titer for 38 of the 41 samples (92.6%). Two samples were anticomplementary in the complement fixation test but gave readable results in the other tests. The complement fixation test was the least sensitive of the procedures examined. The commercial enzyme-linked immunosorbent assay system was the most practical method and offered the highest degree of sensitivity in detecting antibodies to cytomegalovirus.

The possibility of transmitting cytomegalovirus (CMV) via blood donors with latent CMV infection was suggested as early as 1951 (39). An infectious mononucleosis-like illness identified as a postperfusion syndrome was shown to be caused by CMV (18, 19), and convincing evidence for blood as a source of CMV has been reported since that time (12).

Recent studies have shown that up to 25% of exchange-transfused newborns may develop CMV infections (2). In one study (41), 13.5% of infants of CMV-seronegative mothers developed infection when exposed to at least one donor with serological evidence of latent CMV infection. Fatal or serious symptoms developed in 50% of the infected infants who had received more than 50 ml of packed erythrocytes. The use of CMV-seronegative donors virtually eliminated transfusion-acquired CMV infections in this group of subjects.

CMV appears to be associated with the leukocyte-rich fractions of fresh whole blood (16, 21, 38). Evidence also indicates that the granulocyte fraction is more closely associated with CMV than is the mononuclear fraction (16). Complicating the picture is the fact that CMV has been recovered from the erythrocyte, plasma, and serum fractions of blood as well as from cerebrospinal fluid (1, 35). The solutions offered to the problem of transfusion-acquired CMV infection rely primarily on the use of leukocyte-poor blood or the use of donors who have no serological evidence of antibodies to CMV. Of these approaches to the problem, the use of CMV-seronegative blood appears to be the most practical.

The intent of this study was to evaluate and compare several current methods for detecting the presence of antibody to CMV. The main purpose was to determine the most overall practical method for clinical laboratories to screen blood used to transfuse certain high-risk patients. The complement fixation (CF) test was chosen as the standard for comparison because of its long-standing and widespread use. The indirect fluorescent-antibody (IFA) technique, a modified indirect hemagglutination (IHA) test, and a commercially available enzyme-linked immunosorbent assay (ELISA) were chosen as examples of newer techniques that are currently in use and may offer certain advantages over the CF test.

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MATERIALS AND METHODS

Sera selection. The sera used were selected from samples submitted to the diagnostic virology laboratory at Loma Linda University Medical Center for determination of antibody titers to CMV. All sera had been previously tested for CMV antibody by the CF method and were stored frozen at −20°C. Sera were selected to include samples showing no anti-CMV activity, as well as samples representing low, medium, and high titers to CMV.

CF assay. CF tests were performed with a standard microtiter method (31). A commercial antigen prepared from CMV strain AD-169 (lot no. 33015; Flow Laboratories, Inglewood, Calif.) was used. Obtained from the same manufacturer were guinea pig complement (lot no. 31009), hemolysin (lot no. 4788), CMV antigen control (lot no. 33471), and sheep erythrocytes (lot no. A4892). Serum titers were expressed as the reciprocal of the highest dilution in which 70% or more of the sheep erythrocytes were not hemolyzed.

ELISA. Detection of antibody by the CMV Bio-Bead Titration Kit (Litton Bionetics, Kensington, Md.) is based on the principle of solid-phase immunoassay. A magnetic transfer device, supplied by the manufacturer, was used to transfer the solid phase from one reaction mixture to another. All reagents, controls, and equipment for performing the test were supplied in the kit.

Ferrous metallic beads with bound CMV antigen from infected cell cultures were used in the test. Negative control beads had bound antigen prepared from noninfected cell cultures and were run in parallel with specific-antigen beads.

The antibody titer was determined as the highest dilution of serum in which a 1+ to 2+ green color developed with the

antigen-coated bead. The degree of color development was compared with that yielded by the positive control sera at the stated titers. For the purposes of this study, the procedure outlined by the manufacturer was followed, with the single exception that the initial serum dilution was 1:8, not 1:100 as specified in the package protocol.

IFA. The IFA test was performed in a standard manner (37). The AD-169 strain of CMV, cultured on L-645 human lung fibroblasts, was used. L-645 cells were originally obtained from the California State Department of Health Virus and Rickettsial Diseases Laboratory and are maintained in the Loma Linda University Virus Laboratory.

Sera were tested in a serial twofold dilution pattern, from 1:8 through 1:256. Goat fluorescein-conjugated anti-human immunoglobulin G (lot no. 78077; Meloy Laboratories, Springfield, Va.) was used.

A positive reaction was indicated by specific nuclear fluorescence observed by microscopic examination with UV light. The antibody level was expressed as the dilution or titer which maintained a 1+ fluorescence, as determined by the reader. Uninfected cells included in the cell spot preparation served as internal negative controls.

IHA. The IHA procedure was basically performed as outlined by Yeager (40). This procedure differed from earlier IHA tests for CMV (4) in that glutaraldehyde-fixed human type O erythrocytes rather than sheep erythrocytes were used. This method had the advantage that glutaraldehyde-fixed, tanned, sensitized cells could be frozen for long periods until needed for the test. Freshly drawn whole human blood from a donor with type O erythrocytes was mixed with sodium citrate, washed several times in phosphate-buffered saline, and fixed in 1% glutaraldehyde (EM grade; Sigma Chemical Co., St. Louis, Mo.) for 30 min. After fixation, the cells were again washed and could be used immediately or stored frozen at −70°C for up to 6 months. The antigen used was CMV strain AD-169 grown in L-645 cells and prepared by sonic treatment of the infected cell suspension after one cycle of freezing and thawing.

The optimal dilution of antigen was determined by titration against known positive sera, using cells treated with the optimal tannic acid concentration for each cell batch. The optimal antigen dilution was 1:8 in our preparation. The optimal tannic acid concentration was determined for each cell batch by testing known positive and negative sera against cells with tannic acid concentrations in the range of 1:20,000 to 1:320,000 (wt/vol) and sensitized with the optimal dilution of antigen. The optimal tannic acid concentration in our test system was 1:40,000. Once the correct antigen and tannic acid concentrations were determined, the fixed, tanned, sensitized human O erythrocytes and control cells (cells treated in like manner but not sensitized with CMV antigen) were stored frozen until needed for the test.

The cells were added to serial twofold dilutions of serum in 0.15 M phosphate-buffered saline (pH 7.2) containing 1% normal rabbit serum and 0.1 M lysine. The serum dilutions ranged from 1:8 to 1:1,024. The cells were allowed to settle for 45 min at room temperature and then read. The titer was considered to be the highest dilution of serum that caused a 3+ to 4+ agglutination.

RESULTS

Two samples gave low CF titers but were negative in each of the other three test systems. Two samples were anticomplementary (AC) in the CF test. It is interesting to note that one of the AC samples was measured as a high-titered positive by the other test procedures, and the remaining AC sample was shown to be negative.

With the exception of these four samples, all sera that were positive in one test system were also positive in each of the other tests. Conversely, all sera giving negative results in one test gave the same result in each of the other methods. The results given by all tests on these sera were qualitatively the same.

The titers obtained demonstrated the quantitative differences between the four methods. The CF test generally gave lower titers than the other procedures (Fig. 1). The data showed that each of these procedures offered a higher degree of sensitivity than did the CF test, as evidenced by the

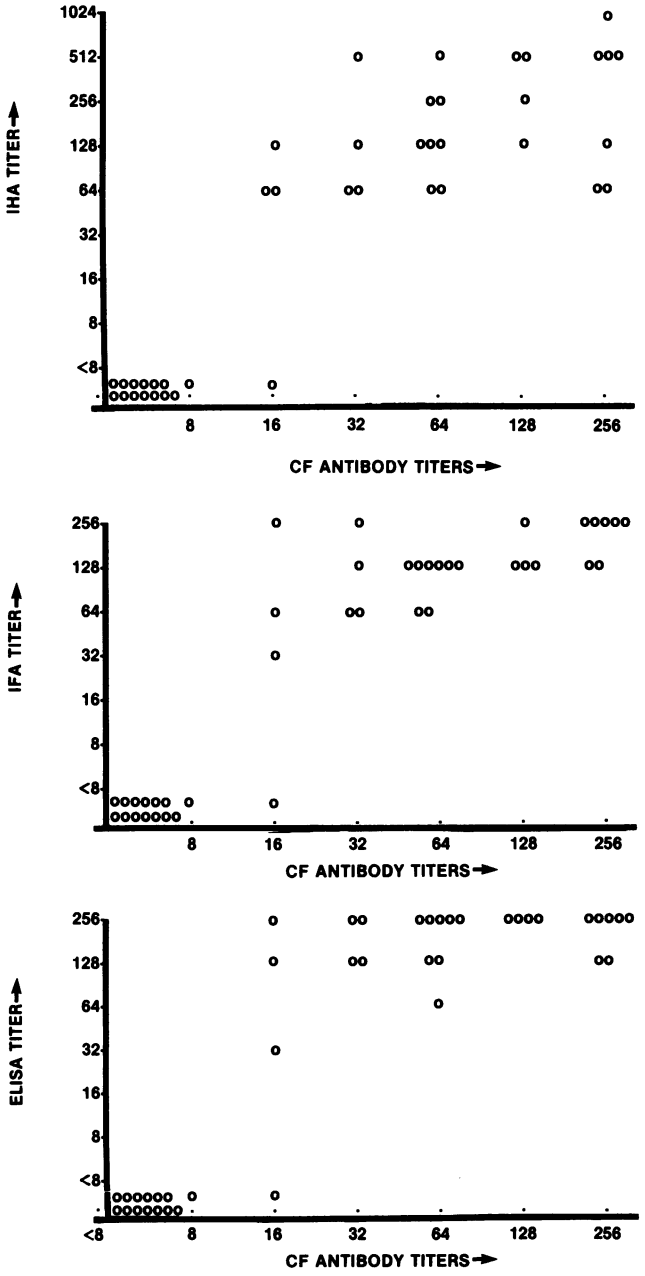

FIG. 1. Comparison of CMV antibody titers obtained in the CF, ELISA, IFA, and IHA tests.

consistently higher titers obtained. The results for the AC sera were excluded from the data plotted in Fig. 1. The IHA titer was tested up to a 1:1,024 dilution; for other methods, a 1:256 dilution limit was used.

The titers obtained by the ELISA, IFA, and IHA tests showed a high degree of correlation (Fig. 2). This correlation was analyzed statistically with Spearman's rank correlation method (Table 1). The high correlation coefficients (r) obtained suggest that the four procedures are measuring similar components of antibody to CMV. The higher degree of correlation between the ELISA, IFA, and IHA results ($r > 0.8$) is in contrast to the lower correlation coefficients obtained when the CF test results were compared with those for the other procedures ($r = 0.4$ to 0.6). Reliable coefficients of correlation ranging from 0.4 to 0.6 may be regarded as indicating a moderate degree of correlation, and values from 0.8 to 1.0 indicate high correlation (13).

It is evident that the titers determined by the ELISA, IFA and IHA assays are higher than the titers from the CF test, as reflected in the geometric mean titers calculated for each test (Fig. 3). The geometric mean titers for the ELISA, IFA, and IHA tests did not differ significantly, but they were all significantly greater than that calculated for the CF test. For the sake of comparison, all titers greater than 1:256 in the IHA test were grouped in the 1:256 category.

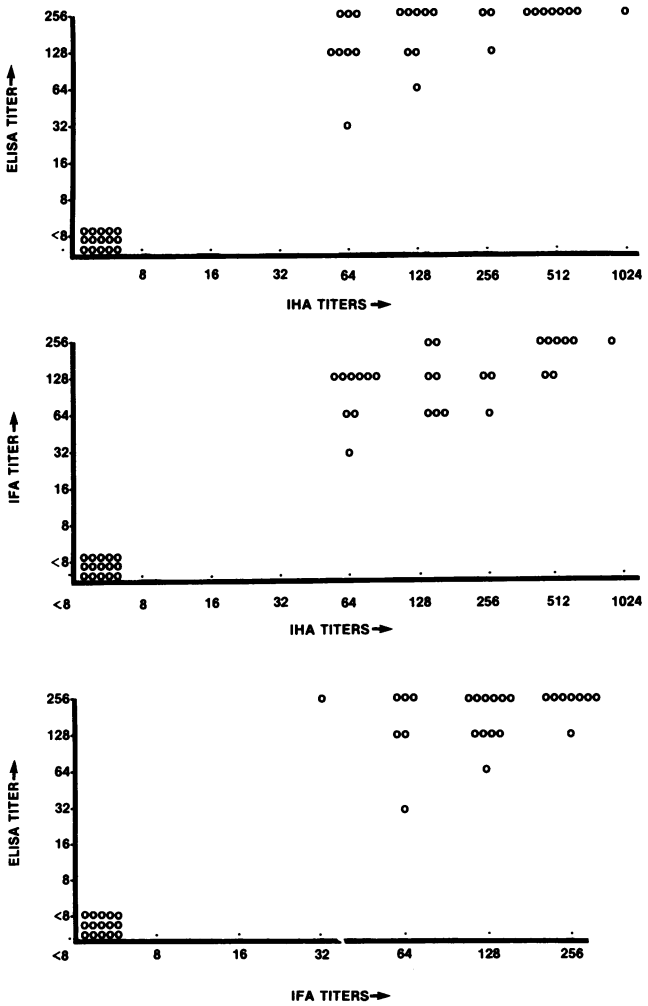

FIG. 2. Comparison of CMV antibody titers obtained in the ELISA, IFA, and IHA tests.

TABLE 1. Correlation between specific antibody titers for CMV detected in 43 sera

Titers compared	r
CF and ELISA	+0.41
CF and IFA	+0.54
CF and IHA	+0.56
ELISA and IFA	+0.89
ELISA and IHA	+0.90
IFA and IHA	+0.87

DISCUSSION

It has been estimated that 66% of the general population of the United States shows serological evidence of CMV infection (34) and that 12% of all blood donors are capable of transmitting the virus (27). In a routine sampling of 1,000 units of blood, 21% had significant titers (1:8 or greater) of CF antibody to CMV (25). The presence of antibody to CMV in a person's serum does not necessarily mean that that person is a carrier of the virus (26), but there is a definite positive correlation between transfusion-acquired CMV infection and the presence of antibody. The amount of blood received is also a factor in establishing CMV infection via blood donors (32, 41).

The CF test, although widely used, may have several limitations as a diagnostic procedure. The sensitivity of the assay is uncertain, and small quantities of antibody may not be detected (15, 22). Although the CF test primarily detects the presence of specific immunoglobulin G (IgG) antibodies, not all subclasses of IgG fix complement (17), and IgM apparently reacts poorly in the CF test used for CMV (23). A negative CF test does not rule out CMV infection, and its usefulness in screening prospective blood donors is limited.

Our results support the conclusions of other investigators that the CF test lacks sensitivity (6, 14). The CF titers were consistently lower than the titers achieved with the other methods. All of the sera that were positive in the ELISA, IFA, and IHA tests were also positive in the CF test, with the exception of the two AC samples. From this standpoint, the CF test was adequate in detecting CMV antibody but did so at a lower level of sensitivity. The AC samples represent another problem with the CF procedure, in that not all sera can be tested by this method. Two samples had CF titers of 1:8 and 1:16, respectively, but were negative in all the other assays. The CF testing was done before the other procedures. It is possible that additional freezing and thawing of these samples adversely affected the ELISA, IFA, and IHA test results. A repeat CF assay was not performed on these two samples due to insufficient sample amounts. It was noted that there was an occasional decrease in titer when certain sera had undergone repeated cycles of freezing and thawing.

It is also possible that the CF test may be detecting specific antibodies that are somehow different from those detected by other assays (14). It has been proposed that "early" antibodies from recent CMV infections may have different antigen-binding properties or that these types of results may reflect differences in the antigens of the test systems (4). The sensitivity of the test may be increased, since a glycine-extracted antigen may result in higher CF titers and CMV antibodies may be detected more frequently (10).

The ELISA technique has been adapted to a variety of test systems for measuring antibody to CMV. The flexibility of the assay has been adequately demonstrated by one investi-

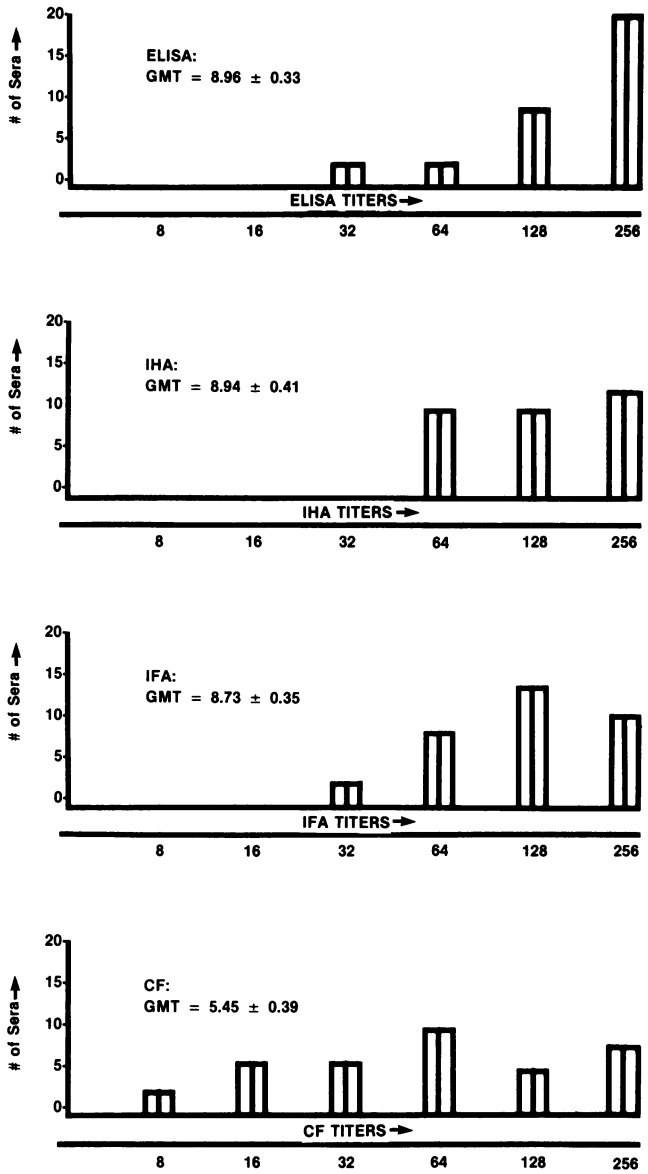

FIG. 3. Distribution and geometric mean titers (GMTs) obtained in the CF, IFA, IHA, and ELISA tests for CMV.

gation, which evaluated seven different ELISA assays from antibody to CMV (43). The ELISA technique has been shown to be effective in detecting and titrating CMV antibody (6). Both IgG and IgM (28, 42) antibodies to CMV have been demonstrated by this procedure. The sensitivity of the test is reported to be high, and the titers obtained compare favorably with those of both the IFA (28) and IHA (8) procedures.

In the present study, the ELISA was found to be easily performed. All controls, reagents, and test materials were available in kit form. As was shown in the results, the ELISA showed the highest degree of sensitivity of the methods examined. The ELISA was the most practical procedure, in our hands, based on ease of handling, speed (less than 4 h), and simplicity of interpretation. Titer comparisons for all four methods were made by diluting sera from 1:8 through 1:256. This was contrary to the manufacturer's recommendation to use dilutions from 1:100 through 1:6,400. The lower dilutions presented no difficulty in interpretation. Two samples gave ELISA titers below 1:100. The manufacturer's indication that a 1:8 CF titer is equal to a 1:100 ELISA titer was not supported by these results.

The IFA test is reported to be more sensitive than the CF test (5, 11) and can be used to effectively screen blood for antibody to CMV (2). CMV infection of human fibroblasts induces an Fc receptor in the cytoplasm of infected cells which may nonspecifically bind IgG. This can result in false-positive reactions with certain samples (37). Nonspecific staining can be minimized by using isolated nuclei for target antigens (29), or the test can be performed as an anticomplement immunofluorescence procedure (20).

The IFA procedure compares well with the IHA test and may be more sensitive (30), and it can be used to measure antibody classes other than IgG (33, 36). Our results indicated a high degree of correlation between results from the ELISA and IHA procedures (Fig. 2). Training individuals to competently read the IFA test may be time-consuming. With experience, it is possible to correctly distinguish between specific nuclear staining and nonspecific cytoplasmic staining. The IFA test is rapid (less than 2 h) and convenient to use once the slides have been prepared.

The IHA test is also a highly sensitive and reproducible procedure. The test is more sensitive than the CF test (4, 9), and IgM antibodies to CMV can be assayed for as well as IgG. The results obtained in IHA system correlated well with those for the IFA and ELISA procedures. The IHA test has an additional advantage in that IHA antibodies are among the first to appear in a CMV infection (24). That the IHA titers were higher than the CF titers may be due to fundamental differences in the antigens used in the tests (3). The modified IHA procedure that we used (40) employs human type O erythrocytes and permits fixed, tanned, sensitized cells to be frozen and stored for up to 6 months. Long-term storage of CMV-sensitized sheep erythrocytes has been one of the major limitations of the standard IHA procedure. Recently, the method of using of freeze-dried sensitized cells has been perfected and is being advocated (7).

The IHA test itself is a rapid, easily performed procedure. The test can be used effectively to screen large numbers of sera for CMV. However, the preparation and pH of the various buffers were found to be critical for proper results. The tannic acid titration was an exacting procedure, and not all of our human O cell batches would work in the test (four out of seven). Each batch of cells reacted differently and had to be titrated separately. It is also of interest that glycine-extracted antigen failed to perform satisfactorily in the IHA procedure. Due to these types of problems, the test was not considered a practical method in our laboratory. Since our study was performed, a commercial preparation for sensitized cells has become available. Many of these problems could be eliminated.

Due to the design of this study, it would be difficult to make a meaningful cost comparison of the methods examined. The IHA and IFA procedures were run in a conventional manner, with all reagents prepared in our laboratory. Commercially prepared reagents are now available for both of these methods. The CF test was also done conventionally, but the reagents were purchased commercially. In contrast, the ELISA was performed with all reagents and materials acquired from the manufacturer.

The true cost per test of all procedures is heavily dependent on the number of samples run at one time. In our study, the number of samples varied with each test run, depending

on when the sera were available and the practical limitations of the laboratory itself. Procedures such as the IHA involved an inordinate amount of preparation, even though the test could be performed very rapidly.

In conclusion, the CF test was found to be the least sensitive method, but it may still be valid because there were two samples that were not positive by any other procedure. The ELISA was shown to be the most sensitive test and also the easiest to use. The results of the IFA and IHA test were comparable with those of the ELISA, but these procedures were shown to be less desirable from a practical standpoint.

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