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Anti-Complement Immunofluorescence Test for Antibodies to Human Cytomegalovirus

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An anti-complement immunofluorescence (ACIF) test that detects human cytomegalovirus (CMV) antigen in the nuclei of infected cells was used for assay of CMV antibodies in human sera. Various factors influencing the sensitivity and specificity of the ACIF test system were investigated, and results were applied to the development of a procedure which could be completed in a relatively short length of time and gave reproducible results. Results obtained in the ACIF test were compared with those obtained in complement fixation, indirect hemagglutination, and neutralization tests, and the ACIF test was shown to be suitable for detection of significant antibody titer rises and stationary levels of CMV antibody. Heterotypic antibody responses were not seen with sera from other human herpesvirus infections. The nonspecific cytoplasmic staining that occurs in indirect immunofluorescence tests for CMV did not occur in the ACIF system, and sera that were anti-complementary in complement fixation tests could be examined satisfactorily by ACIF. Thus, the test is a valuable supplemental or back-up procedure for the serodiagnosis of CMV infection.

The most widely used test for measurement of antibodies to human cytomegalovirus (CMV) has been the complement fixation (CF) test. Although CF is a convenient method for routine testing of large numbers of sera, there are several drawbacks associated with the procedure. Its sensitivity may be lower than that of other antibody assays for CMV, such as neutralization, immunofluorescence-staining, and indirect hemagglutination (IHA) tests (1, 7, 14), and the sensitivity of the CF test depends to a large extent upon the type of antigen preparation employed (2). CMV CF titers in the low range of <1:8 to 1:8 may not be reproducible, making accurate interpretation difficult, and sera with anti-complementary activity present problems in the test system. Also, the test is not suitable for diagnosing recent or congenital CMV infections through the demonstration of virus-specific immunoglobulin M (IgM) antibody.

The IHA test for CMV (2, 4) is sensitive and specific, but is difficult to standardize for use in the routine laboratory diagnosis of CMV. Further, indirect fluorescent-antibody (IFA) tests have not been generally satisfactory for assay of CMV antibody because the immunoglobulin G (IgG) receptors present in the cytoplasm of CMV-infected cells (5, 17) cause nonspecific staining that may give misleading results with negative sera or may obscure the specific nuclear staining produced by CMV antibody.

In efforts to devise a more reliable test for the serodiagnosis of CMV infection, the anti-complement immunofluorescence (ACIF) method, which has been useful for detecting specific nuclear antigens of Epstein-Barr virus (6, 9, 10, 13), was explored. This report describes the development and evaluation of an ACIF procedure for assay of CMV antibodies. It is a sensitive and specific technique that is applicable to routine use in the diagnostic laboratory.

MATERIALS AND METHODS

Cell cultures. Virus was propagated in a line of human fetal diploid lung cells (L-645) developed by J. H. Schieble of this laboratory. Cells were grown on fortified (containing twice the standard concentrations of vitamins and amino acids) Eagle minimum essential medium supplemented with 10% fetal bovine serum.

Virus strains. The AD-169 strain of human CMV was used for the studies. High-titered seed virus preparations with infectivity titers of $\geq 10^7$ plaque-forming units per ml were prepared as described elsewhere (11). Cell cultures for use in the ACIF test were infected at a multiplicity of ≥ 1 plaque-forming unit of virus per cell and harvested after 96 h of incubation at 36°C (3). A simian CMV strain recovered in this laboratory from uninoculated primary rhesus monkey kidney cell cultures was used for certain studies.

ACIF procedure. Based upon the developmental studies described below, the following method was devised for assay of CMV antibodies in human sera.

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Infected cells in a 32-ounce (ca. 0.946-liter) culture bottle were dispersed with trypsin and suspended in 1 to 2 ml of growth medium. Three spots of cell suspension approximately 3 mm in diameter were placed on each clean microscope slide, air dried, and fixed in acetone for 10 min at room temperature; the slides were stored at −70°C.

The IgG fraction of goat antiserum to guinea pig complement C3 (B_1C/B_1A) was obtained from Cappel Laboratories, Inc., Downington, Pa. Protein determination was made by the biuret technique, and the globulins were conjugated with fluorescein isothiocyanate at a ratio of 1 mg of fluorescein to 100 mg of protein. Uncoupled fluorescein was removed from the conjugate by passage through a Sephadex G-50 column, and the conjugate was stored at 4°C. Guinea pig complement was obtained from Microbiological Associates, Bethesda, Md.

Test serum (inactivated at 56°C for 30 min) was diluted in 0.01 M phosphate-buffered saline (PBS; pH 7.2), and complement and conjugate were diluted in Veronal-buffered saline (pH 7.2), 0.1 ionic strength, to stabilize the complement.

Optimal dilutions of complement and conjugate for use in the ACIF test were determined by box titrations of varying dilutions of each reagent against CMV-infected cells treated with appropriate dilutions of known positive sera (and known negative sera for control purposes). Complement was tested at dilutions from 1:10 through 1:60 and conjugate at dilutions from 1:20 through 1:100. Complement at a dilution of 1:40 and conjugate at a dilution of 1:50 or 1:60 generally gave optimal results.

The ACIF procedure used to demonstrate CMV antibody in human sera consisted of the following steps. CMV-infected cells fixed on glass slides were treated with serial twofold dilutions of test serum (inactivated at 56°C for 30 min), starting with a 1:8 dilution. The first three dilutions were also applied to fixed, uninfected cells for control purposes. After incubation for 20 min at 37°C in a humidified chamber, slides were rinsed for 5 min in PBS. An optimal dilution of complement was then added, and incubation was conducted for 20 min at 37°C in a moist chamber. After a 5-min rinse in PBS, an optimal dilution of fluorescein-conjugated anti-guinea pig complement was added, and incubation was conducted for 20 min at 37°C in a moist chamber. After a 5-min rinse, the slides were allowed to air dry, were mounted in 25% glycerol in PBS, and were examined under ultraviolet illumination. A human serum known to be positive for CMV antibody by CF and ACIF and a human serum found to be free of CMV antibodies by CF, ACIF, and neutralization tests were included for controls. Specific nuclear staining (Fig. 1) was taken as evidence of the presence of CMV antibody in the test serum. The antibody end point was the highest dilution showing specific nuclear fluorescence in CMV-infected cells.

Other CMV antibody assays. Plaque-reduction neutralization (11), IHA (1, 4), IFA (12), and CF (8) assays for CMV antibody are described elsewhere. For

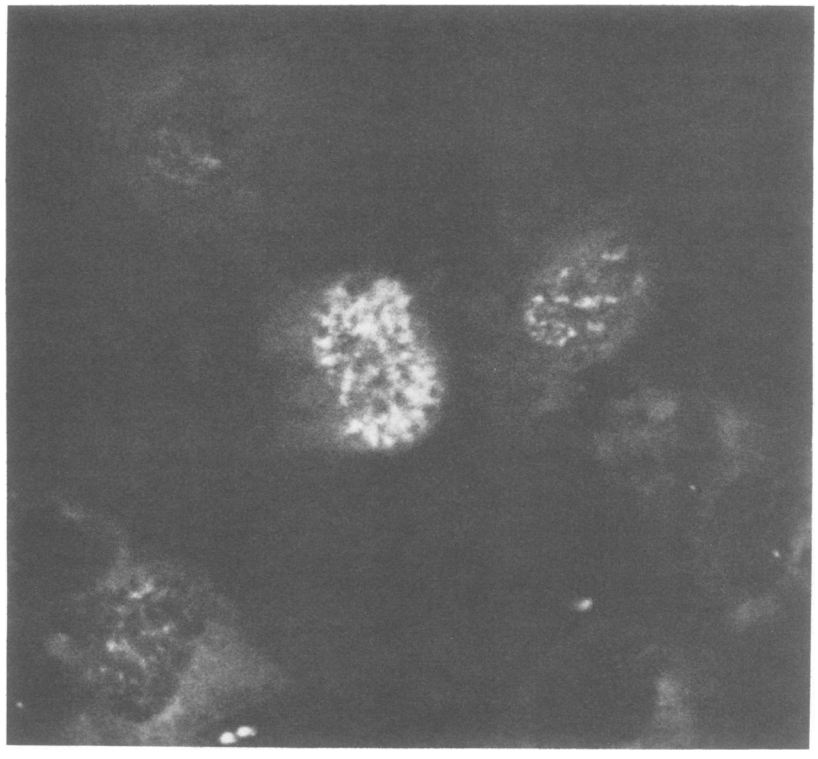

FIG. 1. *ACIF staining of CMV antigen in nuclei of infected cells.*

CF tests, antigens prepared both by freezing and thawing and by extraction with glycine buffer (2) were employed.

Sera examined. Human sera used in these studies were from our diagnostic files. For the initial phases of the study, sera were selected on the basis of positive or negative CMV CF results, or on the basis of being "problem" specimens in the CF test. For the final evaluation of the ACIF test, sera with various levels of CMV CF activity were coded by a statistician for a "blind" study.

RESULTS

Developmental studies. Various factors affecting the sensitivity and specificity of the ACIF system with CMV were investigated as a basis for establishing a routine procedure.

The concentration of complement used in the system was found to influence the specificity of the test. This is illustrated in Table 1, which shows results of titrating varying dilutions of complement with constant dilutions of sera both positive and negative for CMV antibodies and with a PBS control. With higher concentrations of complement, nonspecific staining was seen with the negative serum and the PBS control. This is probably due to attachment of the guinea pig IgG to the receptors in the cytoplasm of CMV-infected cells (5, 17). With higher dilutions of complement, however, nonspecific cytoplasmic staining was virtually eliminated, and the specific nuclear staining produced by the positive serum was better defined. Similar results were obtained using hamster antiserum against CMV. These findings illustrate the importance of pretitrating each lot of complement to be used in the ACIF system so that a dilution giving optimal sensitivity and specificity can be selected.

The same cells, test sera, and PBS control used in the ACIF experiments shown in Table 1 were also tested at the same time by IFA staining, and, in contrast to the minimal cytoplasmic staining seen with optimal dilutions of complement in the ACIF test, the CMV-positive serum showed strong cytoplasmic staining, which made definition of nuclear staining difficult; the negative serum gave 2+ cytoplasmic staining; and the PBS control also showed a low level of cytoplasmic staining. This illustrates the improved specificity of ACIF staining for CMV antibody over that of IFA staining.

As would be expected, the specificity of the ACIF test was also influenced by the concentration of the anti-guinea pig complement conjugate, and higher concentrations of this reagent gave nonspecific staining; therefore, it was important to titrate the conjugate in the presence of optimal concentrations of complement to select an appropriate working dilution that gave clear-cut specific nuclear staining and no appreciable cytoplasmic staining.

In efforts to shorten the time required for completion of the ACIF test, and, thus, to make it more useful for routine application in the diagnostic laboratory, studies were conducted to determine the shortest incubation periods that could be used without sacrificing sensitivity of the test. Results obtained with incubation times of 20 min for each of the three reaction steps gave results comparable to those obtained with longer incubation periods (Table 2), and these times were adopted for routine use.

Because of a recent report (15) that cells infected with a simian strain of CMV did not give nonspecific cytoplasmic fluorescence in IFA tests with human sera, parallel tests were con-

TABLE 1. *Complement titration in the ACIF test using human antisera*[a]

Complement dilution	Staining reactions with[b]:		
	Pos. serum	Neg. serum	PBS control
1:10	3–4+ nucleus (whole cell)	Negative nucleus 1–2+ cytoplasm	Negative nucleus 1–2+ cytoplasm
1:15	2+ nucleus and cytoplasm	Negative nucleus 1+ cytoplasm	Negative nucleus 1+ cytoplasm
1:20	2–3+ nucleus 1+ cytoplasm	Negative nucleus ± cytoplasm	Negative nucleus ±–+ cytoplasm
1:30	2–3+ nucleus ± cytoplasm	Negative nucleus ± cytoplasm	Negative nucleus ± cytoplasm
1:40	2–3+ nucleus ± cytoplasm	Negative nucleus ± cytoplasm	Negative nucleus ± cytoplasm

[a] Conjugate used at optimal dilution of 1:60.

[b] 3+, Brightest stain. Pos, Positive serum (1:256 by CF), and Neg, negative serum (<1:8 by CF and neutralization), both used at 1:30 dilution.

TABLE 2. *Comparison of incubation times used in the ACIF test*

Time[a]	Antiserum dilution[b]					
	8	16	32	64	128	256
Expt 1						
20-20-20	2-3+	2-3+	2-3+	2-3+	2+	1-2+
45-45-30	2+	2-3+	2-3+	2+	1-2+	1-2+
Expt 2						
20-20-20	1+	1+	1+	2+	2+	2+
45-45-30	1-2+	1-2+	1-2+	2+	2+	1-2+

[a] Time in minutes for each of the three reaction steps in the ACIF test.

[b] Nuclear staining, 3+ being the brightest.

ducted comparing the sensitivity and specificity of ACIF tests using the AD-169 strain of human CMV and a simian CMV strain from this laboratory. Representative results (Table 3) show that CMV antibody could be detected only in the higher-titered serum, using the simian virus-infected cells. Further, differentiation between nuclear and cytoplasmic staining was not clear-cut in the ACIF test with simian virus-infected cells, and the degree of cytoplasmic staining resembled that seen in IFA tests. It is possible that different strains of simian CMV vary in their suitability for use in immunofluorescence tests for detection of antibody in human sera.

Cells infected with the AD-169 strain of CMV at a multiplicity of infection of ≥1 plaque-forming unit per cell were harvested at various times after infection and examined in the ACIF test to determine the optimal time for harvest of cells to be used as a source of antigen in this test system. CMV antigen development was found to be maximal at 96 h after infection, as has also been shown by direct and indirect immunofluorescence staining and radioimmunoassay (3).

Comparisons were made using PBS and Veronal-buffered saline as diluents for the complement and conjugate. Veronal-buffered saline appeared to be a more stabilizing buffer, since reagents in this diluent gave brighter specific nuclear fluorescence than did those diluted in PBS. Therefore, Veronal-buffered saline was adopted for routine use in the CMV ACIF test.

Comparative sensitivity and specificity of ACIF test for detection of CMV antibodies in human sera. After completion of the basic developmental studies, the ACIF test was compared with neutralization, IHA, and CF tests for sensitivity and specificity in detecting CMV antibody. Thirty sera with varying levels of CF antibody were examined under code, and titers obtained in the different test systems are compared in Table 4. For the positive sera, titers in the various test systems varied widely; this is probably a reflection of the fact that the tests detect different classes of antibody to CMV. Sera showing low CF titers with glycine-extracted antigen and negative titers with the freeze-thaw CF antigen were included as "problem" sera for which interpretation of CF results would be difficult. In most instances, the ACIF and other tests confirmed the presence of antibody in the sera. For the most part, titers obtained by ACIF were equal to or slightly higher than those seen with the glycine-extracted CF antigen, and they were markedly higher than those seen with the freeze-thaw CF antigen. ACIF titers were generally equal to or slightly higher than neutralizing antibody titers, but they tended to be lower than titers obtained in IHA tests. Two sera had low titers by ACIF and by CF with a glycine-extracted antigen and titers of <1:8 by IHA, and one serum had a high titer by IHA, a negative titer by ACIF, and negative or low titers in the other assays. CF tests with glycine-extracted antigen detected 25 positive sera among the 30 tested; CF tests with freeze-thaw antigen detected 11 positive sera; IHA demonstrated 20 positive sera; neutralization tests detected 18 positive sera; and ACIF demonstrated 22 positive sera. Thus, the ACIF test was comparable to or more sensitive than the other tests for detecting the presence of CMV antibody.

Five sera that were anti-complementary and did not give reliable results in the CF test were examined by the ACIF test, and specific CMV antibody, which was confirmed by neutralization

TABLE 3. *Comparison of fluorescent-antibody staining by the ACIF method of cells infected with AD-169 or simian CMV*

Serum	Serum dilution	AD 169 CMV	Simian CMV	Uninfected cells
Positive serum #1 (1:64 CF)	1:8	2+ nucleus	4+ difficult to define structure	±
	1:16	2-3+ nucleus	3-4+ nucleus & cytoplasm	−
	1:32	+	3+ nucleus & cytoplasm	−
Positive serum #2 (1:8 CF)	1:8	2-3+ nucleus (bright)	Negative (some debris)	−
	1:16	1+ nucleus	Negative	−
	1:32	1+ nucleus	Negative	−
Negative serum #3 (<1:8 CF)	1:8	± cytoplasm	Negative (± cytoplasm)	−
	1:16	+ cytoplasm	Negative	−
	1:32	Negative	Negative	−

TABLE 4. *Comparison of CMV antibody titers obtained in ACIF test with those obtained in other test systems*

Serum no.	CF[a]		IHA	Neutralization	ACIF
	GE	FT			
1	<8	<8	<8	<8	<8
2	<8	<8	<8	<8	<8
3	<8	<8	<8	<8	<8
4	<8	<8	<8	<8	<8
5	<8	<8	<8	<8	<8
6	8	<8	<8	<8	8
7	8	8	256	16	16
8	8	<8	16	<8	16
9	8	<8	128	32	16
10	8	<8	<8	<8	<8
11	8	<8	<8	<8	32
12	8	<8	<8	<8	<8
13	8	<8	1,024	8	<8
14	8	<16	64	64	64
15	8	<8	128	<8	16
16	16	<8	256	16	16
17	16	<8	1,024	64	16
18	32	<8	256	16	32
19	32	<8	128	8	16
20	64	32	>4,096	128	32
21	64	8	256	128	64
22	64	8	256	64	256
23	64	8	512	128	32
24	64	16	512	128	512
25	128	<8	NS	64	32
26	256	128	>4,096	64	1,024
27	256	16	512	64	256
28	256	32	>4,096	64	128
29	256	64	2,048	<8	64
30	256	256	>4,096	256	512

[a] GE, CF antigen prepared by extraction of infected cells with glycine buffer; FT, CF antigen prepared by freezing and thawing infected cells.

and IHA tests, was demonstrable in each. Thus, the ACIF method can be used for CMV antibody assay of certain sera that are unsatisfactory for CF tests.

Diagnostic reliability of ACIF test for CMV antibody. The ability of the ACIF test to detect diagnostically significant increases in homologous antibody titer in CMV infections was compared with that of the CF and IHA tests, and the results of representative experiments (Table 5) illustrate the diagnostic capability of the ACIF test system.

Although infections with herpes simplex or varicella-zoster viruses have not been observed to elicit heterotypic antibody titer rises to CMV, the specificity of the ACIF test for CMV was evaluated by examining three serum pairs from patients with herpes simplex virus or varicella-zoster virus infections who showed CF antibody titer rises to the heterotypic as well as the homotypic virus and who also had CF antibody to CMV. CMV antibody titers detected by the ACIF remained stationary during the development of antibody to herpes simplex and varicella-zoster viruses. This indicates that other human herpesvirus infections are not likely to cause misleading results in the ACIF test for CMV antibody.

DISCUSSION

These studies have shown that an ACIF test similar to those used for detection of antibody to certain other human herpesviruses (6, 9, 10, 13, 16) can be applied for assay of CMV antibodies against the virus-specific antigens present in the nuclei of infected cells. The ACIF test for CMV antibodies was developed with a view toward making it applicable for routine use in a diagnostic laboratory, rather than as a research tool.

Guinea pig complement, instead of human complement, was used in the CMV ACIF test, because of possible difficulties in obtaining human sera for complement source that were free from antibodies to CMV or to other human agents, which might give misleading results. Guinea pig complement from a commercial source (pretested for absence of antibodies to certain human viral, rickettsial, and chlamydial; agents) proved to be satisfactory for use in the ACIF test, as did commercially available IgG against guinea pig complement.

Relatively short incubation and washing steps were adopted to shorten the time required for performing the test, and thus to make it more

TABLE 5. *Ability of ACIF test to demonstrate diagnostically significant rises in CMV antibody titer*

Patient	CF[a]		ACIF	IHA
	FT	GE		
Me	<8	<8	<8	64
	128	≥256	128	256
St	<8	<8	<8	16
	32	128	512	512
Ra	8	<8	256	128
	256	≥256	1,024	4,096
Go	16	32	64	4,096
	64	128	256	8,192
Hi	16	64	256	64
	≥128	512	4,096	2,048
Ma	8	8	16	2,048
	16	64	64	4,096

[a] See Table 4, footnote *a*.

useful for routine use. Comparative studies showed that the shorter test was as sensitive and specific as tests using longer incubation and washing steps. The total time required for performance of the ACIF test is approximately the same as that for an IFA test, even though there is an additional step in the ACIF procedure.

Both complement and the anti-complement conjugate must be pretitrated to determine optimal dilutions for use in the test; the use of these reagents at too-high concentrations will cause nonspecific cytoplasmic staining. It was also found important to avoid drying of the slides between steps in the procedure, since drying before completion of the test resulted in staining of low intensity.

A major advantage of the ACIF test over IFA tests for detection of CMV antibody is that nonspecific cytoplasmic staining is not a problem in the former system if optimal concentrations of reagents are used. This is likely due to the fact that complement attaches only to antigen-antibody complexes, which are present in the nucleus of infected cells treated with viral antibody, and the IgG attached nonspecifically to the receptors in the cytoplasm is not labeled in the reaction.

The ACIF test was clearly shown to be as reliable as the more time-consuming and expensive neutralization and IHA tests for detection of CMV antibody. It was equally or slightly more sensitive than the CF test with a glycine-extracted antigen, and markedly more sensitive than the CF test with a freeze-thawed antigen. Whether the ACIF test is capable of detecting CMV-specific IgM antibody remains to be established; this may prove to be a limitation of the technique, since tests involving fixation of complement are generally relatively insensitive for detecting viral IgM antibody.

In addition to its general usefulness in detecting CMV antibody and demonstrating diagnostically significant rises in CMV antibody titer, the ACIF test is particularly valuable as a back-up procedure to aid in the interpretation of low CF titers, and for testing sera that are anticomplementary in CF tests. The test can be readily incorporated into the routine operations of a viral diagnostic laboratory.

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