

Anticomplement Immunofluorescence Test That Uses Isolated Fibroblast Nuclei for Detection of Antibodies to Human Cytomegalovirus

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Cytomegalovirus antibodies were measured in human sera by a nuclear anti-complement immunofluorescence test that used as antigen the isolated nuclei of virus-infected fibroblast cells lysed in distilled water. The method exhibited less nonspecific fluorescence than either a conventional whole-cell anticomplement immunofluorescence test or an indirect fluorescent antibody test applied to the same isolated nuclear substrate. The assay detected 97.5% of 40 antibody-positive sera, compared with 92.5 and 90% detection rates by indirect hemagglutination and complement fixation, respectively. In addition, antibody titers obtained by this technique were significantly higher than those obtained by either indirect hemagglutination ($P < 0.02$) or complement fixation with a glycine-extract antigen ($P < 0.001$).

Although the complement fixation (CF) test is widely used to detect antibodies to cytomegalovirus (CMV), newer tests such as indirect hemagglutination (IHA) and indirect immunofluorescent antibody (IFA) assays are less cumbersome and time-consuming, offer greater sensitivity, and are not affected by anticomplementary sera (1, 3, 5). The utility of the IFA test has been questioned, however, since the discovery that immunoglobulin G receptor sites, induced in the cytoplasm of CMV-infected cells, may produce false-positive results due to nonspecific staining (8, 10, 17). Although specificity may be achieved by accepting as positive only those cells exhibiting specific intranuclear staining (3), an assay devoid of nonspecific fluorescence is clearly desirable. The anticomplement immunofluorescence (ACIF) test avoids cytoplasmic staining by virtue of the fact that complement is fixed only by intranuclear CMV antigen-antibody complexes and not by antibody nonspecifically bound to cytoplasmic receptors (10, 11).

Recent work in our laboratory demonstrated that lysis of CMV-infected fibroblast cells in distilled water yields a uniform population of morphologically intact nuclei containing typical cytomegalic inclusions. This finding prompted us to evaluate these naked nuclei as substrates for a CMV immunofluorescent antibody assay. As an added control against nonspecific fluorescence, it seemed reasonable to apply the ACIF technique to these cytoplasm-free nuclei. In this report, we describe our results with ACIF and conventional IFA assays on naked nuclei, con-

trast them with the results of a standard whole-cell ACIF technique, and compare the sensitivity and specificity of the nuclear ACIF (NACIF) test with CF and IHA assays of CMV antibody in human sera.

MATERIALS AND METHODS

Sera. Initial studies to compare various immunofluorescent techniques were performed on sera chosen from diagnostic specimens stored at -20°C in the Clinical Virology Laboratory, Mount Zion Hospital and Medical Center. All had been previously tested for CMV CF antibody by a microtiter technique (12) at the San Francisco Department of Public Health and were selected to include antibody-positive and antibody-negative sera. Subsequent studies with the NACIF test were performed on 100 sera obtained from nursing personnel at Stanford University Medical Center. All specimens were coded to permit subsequent serological testing on a single-blind basis.

Cell cultures and media. Virus was propagated in a line of human diploid foreskin fibroblasts developed in this laboratory, and cells used were taken between passages 8 and 10. Samples of whole cells had been cultured in human embryonic lung, cynomolgus monkey kidney, and Hep-2 tissue cultures (Flow Laboratories, Inc., McLean, Va.), and in diphasic mycoplasma medium (Bakte Bennett, Berkeley, Calif.) and were found to be free of endogenous viral and mycoplasma contamination. Cells were grown in Eagle minimal essential medium containing gentamicin (50 $\mu\text{g}/\text{ml}$), amphotericin B (2.5 $\mu\text{g}/\text{ml}$), and 10% heat-inactivated fetal bovine serum (FBS).

Virus. The AD-169 strain of human CMV was obtained from the American Type Culture Collection, Rockville, Md. Infected fibroblast cells showing 80 to 90% cytopathogenic effect were trypsinized, and the

cells from one 75-cm² plastic flask (Falcon Plastics, Oxnard, Calif.) were added to fresh, confluent fibroblast monolayers in two additional flasks. Flasks were incubated at 37°C and harvested by trypsinization after 96 h, at which time cytopathic effect was evident in 90 to 100% of cells.

Preparation of whole cells for immunofluorescence testing. Trypsinized infected cells were washed twice in phosphate-buffered saline and suspended in phosphate-buffered saline containing 2% FBS. A sufficient volume of phosphate-buffered saline (approximately 5 ml per flask) was used to provide a concentration of 10⁶ cells per ml. Drops of the cell suspension were placed in each of eight 5-mm circles on glass slides, which were then air dried, fixed in acetone for 10 min at room temperature, and stored at -70°C. The cell density in each circle was such that cells were closely spaced but not overlapping.

Preparation of naked nuclei for immunofluorescence testing. Trypsinized CMV-infected cells were suspended in 10 ml of sterile distilled water containing 2% FBS, and the mixture was centrifuged at 500 × *g* for 10 min. Supernatant fluid was removed; twice more the pellets were resuspended in distilled water containing 2% FBS, and the mixture was centrifuged, as just described. Nuclear pellets from each flask were finally suspended in 4 to 5 ml of distilled water with 2% FBS, and slides were prepared as for whole cells.

Immunofluorescence testing. (i) IFA assay. A drop of each serum dilution (1:8 through 1:1,024) was applied to slide-fixed nuclei and allowed to react for 20 min at 37°C in a humidified chamber. After two 5-min rinses in phosphate-buffered saline, a drop of polyvalent fluorescein-conjugated rabbit antiserum to human immunoglobulins A, G, and M (Miles Laboratories, Elkhart, Ind.) was applied to each spot. Prior titration had established an optimal dilution of 1:20 for this antiserum. Slides were incubated as above, washed in two changes of phosphate-buffered saline and air dried.

(ii) ACIF. Whole-cell ACIF and naked NACIF assays were performed by the method of Kettering et al. (11). Guinea pig complement was obtained from Microbiological Associates, Bethesda, Md., and fluorescein-labeled goat antiserum to guinea pig complement C3 (B₁C/B₁A) was obtained from Cappel Laboratories, Cochranville, Pa. Prior titration had established the optimal dilution for complement to be 1:10 and for conjugate to be 1:15.

All fluorescent preparations were examined without cover slips under a Zeiss epifluorescence microscope. Nuclear fluorescence was graded 0, ±, 1+, 2+, or 3+. Antibody titer was determined as the highest serum dilution producing 1+ specific nuclear fluorescence. Uninfected control cells or nuclei were included in each run, as were sera determined to be CMV antibody-positive and antibody-negative by prior CF and NACIF testing.

IHA test. IHA titers were performed by the method of Yeager (19) with tanned, glutaraldehyde-fixed human O erythrocytes in a buffer containing 0.1 M lysine.

CF test. A microtiter CF test (12) that used glycine-extracted CMV antigen (6) was performed at the Viral and Rickettsial Disease Laboratory, State of California

Department of Health Services, Berkeley, Calif.

Statistical analysis. Antibody titers obtained by CF, IHA, and NACIF assays were compared by paired Student's *t*-test with log_e transformation used to correct for unequal variances (7).

RESULTS

Comparison of immunofluorescent assays. Eight sera were defined as positive (six) or negative (two) for the presence of CMV antibody on the basis of concordant results from CF and IHA testing. Results of the three immunofluorescent antibody assays performed on these specimens are shown in Table 1. In general, titers obtained by the whole-cell ACIF test were equal to or lower than the corresponding CF titers. Some degree of nonspecific cytoplasmic fluorescence was noted in the whole-cell ACIF test which, in the case of the two antibody-negative sera, made accurate interpretation impossible. Correlation of the NACIF results with those of the CF and IHA tests was 100% in this small sample. The nuclear IFA (NIFA) test, on the other hand, yielded one presumed false-positive result (serum 2, Table 1). Moreover, the intensity of fluorescence in the NIFA assay was considerably duller, and the endpoints were more difficult to determine, than in the NACIF test. Parallel titrations of 19 additional sera by both NACIF and NIFA revealed consistently higher titers with the former technique (Fig. 1). In light of these findings, the whole-cell ACIF and the NIFA assays were abandoned, and further immunofluorescence studies were confined to the NACIF technique.

Sensitivity and specificity of the NACIF test. Table 2 summarizes the results of NACIF, IHA, and CF testing on 100 sera from nursing personnel. Concordance of results was 97% between NACIF and CF, 97% between IHA and CF, and 96% between NACIF and IHA. Overall,

TABLE 1. Comparison of CMV antibody titers obtained in three fluorescent assays with those obtained by CF and IHA

Serum no.	Titer obtained by:				
	CF	IHA	ACIF	NACIF	NIFA
1	<8	<8	**	<8	<8
2	<8	<8	**	<8	8-16
3	16	64	16	32	64
4	64	64	16	256	32
5	64	512	64	256	512
6	128	16	16	512	64
7	128	256	32	128	64
8	512	128	16	≥1,024	≥1,024

** , Equivocal results due to nonspecific cytoplasmic fluorescence.

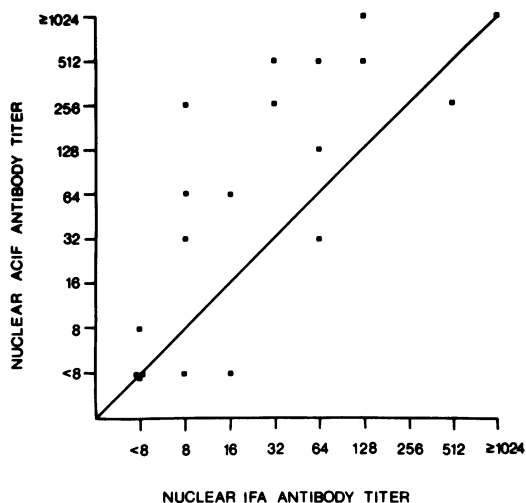


FIG. 1. Comparison of CMV antibody titers obtained by NIFA and NACIF in 19 human sera.

TABLE 2. Results of testing 100 sera for CMV antibody by CF, IHA, and NACIF assays

Results	No. of sera tested	Assay ^a		
		CF	IHA	NACIF
Concordant	35	+	+	+
	60	-	-	-
Discordant	2	-	-	+
	1	-	+	-
	1	-	+	+
	1	+	-	+

^a +, Antibody detected; -, antibody not detected.

95 of 100 sera demonstrated copositivity or conegativity in all three assays. A total of 40 sera were CMV antibody positive by a least one of the three assay methods. Of these sera, the NACIF test detected 39 (97.5%), the IHA test detected 37 (92.5%), and the CF test identified 36 (90%). All sera positive by CF were also positive by NACIF; however, one CF-positive serum sample gave a negative result by IHA.

Among the 35 sera positive in all three assays, the geometric mean titers (\pm standard error of the mean) were 502.9 (± 71.0) for NACIF, 232.2 (± 31.0) for IHA, and 37.7 (± 7.7) for CF. These differences are significant for both NACIF and IHA compared with CF ($P < 0.001$) and for NACIF compared with IHA ($P < 0.02$). Figure 2 compares the results of 40 sera yielding positive titers in either the NACIF or IHA assay. NACIF titers exceeded those of the IHA test by a fourfold or greater dilution in 16 sera, whereas IHA titers exceeded NACIF titers by fourfold or

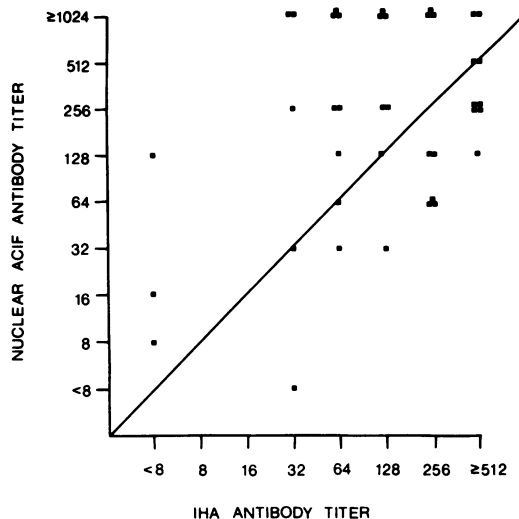


FIG. 2. Comparison of CMV antibody titers obtained by NACIF and IHA assays in 40 human sera. Not included are 60 sera with negative titers (< 8) by both tests.

more in only 6 sera. Thus, the NACIF, IHA, and glycine-extracted antigen CF tests were of comparable sensitivity in detecting CMV antibody, whereas the NACIF test yielded titers significantly higher than those of the other two assays.

DISCUSSION

The whole-cell ACIF test has been utilized for the detection of CMV antibodies to avoid the nonspecific cytoplasmic fluorescence which plagues other CMV IFA assays (10, 11). It was surprising, then, to observe in this study a degree of cytoplasmic fluorescence in the whole-cell ACIF test sufficient to interfere with the interpretation of CMV antibody-negative sera. Kettering et al. (11) noted appreciable cytoplasmic staining in their ACIF assay when either complement or anticomplement conjugate was used at low dilutions. In the present study, use of complement or conjugate at dilutions greater than 1:10 and 1:15, respectively, resulted in pronounced diminution of specific nuclear fluorescence. Lot to lot variations in the potency of fluorescent conjugates are commonly encountered, and heterologous antibodies frequently contaminate lots of "monospecific" antisera (4, 15). It is therefore possible that low-titered reagents, when used in low dilutions to obtain optimal nuclear fluorescence, may yield unacceptably high degrees of nonspecific cytoplasmic staining. It is notable that nonspecific fluorescence was not observed in the NACIF test, despite the fact that both complement and conjugate were used at relatively low dilutions. Exten-

sive adsorption procedures or sensitive radial immunodiffusion testing could be performed on lots of commercial reagents to assure their specificity (15). Such techniques are beyond the capability of most diagnostic laboratories and would, in any event, markedly limit the speed and ease of performance of immunofluorescent assays.

Stagno et al. (16) recently reported the use of purified nuclei as an antigen to detect CMV antibody by a standard IFA assay. Their technique for obtaining nuclear preparations included hypotonic lysis and Dounce homogenization of CMV-infected cells with subsequent separation of nuclei from cellular debris by centrifugation through a viscous cushion of 22.5% Ficoll 400. It is possible that this elaborate procedure separates cytoplasmic debris from nuclei more completely than does the simplified technique reported here. The presumed false-positive result obtained with serum 2 (Table 1) in the NIFA test may represent incomplete removal of cytoplasm (and cytoplasmic immunoglobulin receptors) from the nuclear preparation. As noted above, however, nonspecific fluorescence was not observed when the ACIF technique was applied to naked nuclei. Of 100 sera, 2 gave positive NACIF titers which were not confirmed by either IHA or CF testing (Table 2). These results may represent false-positive reactions by NACIF. Alternatively, they may reflect a greater sensitivity of the NACIF test or the fact that immunofluorescent assays measure different classes of CMV antibody than do CF or IHA tests (9, 11).

Recent reports have illustrated the serious pathogenic potential of CMV when transmitted to susceptible individuals via blood transfusions (14, 18) or organ transplants (2, 13). The use of CMV antibody-negative blood has been shown to decrease the incidence of acquired CMV infection in transfused neonates (A. S. Yeager, F. C. Grumet, E. B. Hafleigh, and C. G. Prober, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 19th, Boston, Mass., abstr. no. 1041, 1979). It is anticipated that organ and blood donor screening for CMV antibody status will become more widely employed in an attempt to limit these acquired infections. In this setting, there is great need for a CMV antibody assay that is sensitive, specific, and, above all, rapidly and easily performed. CF tests are time-consuming and are often not sufficiently sensitive to fulfill these criteria (3). The IHA test is sensitive and specific but is difficult to standardize (5, 11) and suffers from a limited shelf life of the indicator erythrocytes, unless liquid nitrogen storage facilities are available (19). IFA assays

are limited by nonspecific cytoplasmic staining (8, 10, 17). Both the whole-cell ACIF test (11) and the purified nuclear IFA assay of Stagno and co-workers (16) attempt to circumvent this problem. The NACIF assay described here employs both of these approaches to eliminate nonspecific cytoplasmic fluorescence. It offers the advantage of a simplified and less time-consuming method of extracting nuclei from cells, and it markedly reduces the variable degree of cytoplasmic staining encountered in the whole-cell ACIF assay (11).

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