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Cytomegalovirus Antigenic Heterogeneity Can Cause False-Negative Results in Indirect Hemagglutination and Complement Fixation Antibody Assays

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Cord sera and antepartum maternal sera from three congenitally cytomegalovirus (CMV)-infected infants and their mothers were CMV seronegative (titer, <8) in a complement fixation (CF) assay with a glycine-extracted CMV AD169 antigen; sera from two of the infants and mothers were also seronegative in a commercial indirect hemagglutination (IHA) assay with AD169 antigen. In tests with their own CMV isolates propagated and made into glycine-extracted CF antigen, all were seropositive. When 108 random cord sera were assayed for CF antibody with AD169, Davis, and A antigens (A is a locally derived antigen from one of the above infants), 44 were seropositive and 54 were seronegative for all three antigens. Of the remaining 10 sera, 4 were positive for A only, 3 were positive for A and Davis only, 2 were positive for Davis and AD169 only, and 1 was positive for AD169 only. All 10 were positive when a mixture of all three antigens was used. The IHA assay with AD169 antigen was positive with only 4 of these 10 sera. These results suggest that up to 6% of sera may be misclassified as seronegative in the CF and IHA assays if only a single antigen is used.

Cytomegalovirus (CMV) serologic testing has recently been used as a means of assessing relative risks for the transmission and acquisition of congenital, transfusion-acquired, and transplant-associated CMV infections and predicting the severity of such infections in high-risk populations. Yeager demonstrated that blood products that were CMV seronegative in an indirect hemagglutination (IHA) assay were associated with a statistically lower incidence of symptomatic acquired CMV infections among premature infant recipients (19). Stagno demonstrated that maternal CMV seropositivity early in pregnancy does not prevent congenital infections but does decrease the risk of symptomatic congenital infections (12). Numerous investigators have demonstrated that CMV-seronegative recipients of kidney, heart, and bone marrow transplants are much less likely to develop the life-threatening complication of a disseminated CMV infection if the donor organs also come from a CMV-seronegative individual (10).

The complement fixation (CF) test for CMV antibody was the first widely used CMV serologic assay, but it has not been widely used for the mass screening of blood products or organ donors because of the requirement for overnight incubation, the need for meticulous cross-standardization of biologic reagents, interference by anticomplementary activity, insensitivity for the detection of immunoglobulin M (IgM)-specific CMV antibody, and questionable sensitivity at higher titers. Other techniques, such as the IHA assay, that avoid many of these difficulties have been more widely used for screening. Although many different serologic techniques have been used for CMV, most use only a single strain of CMV (usually a standard laboratory strain such as Davis or AD169) as the antigen. If a person has been infected with a wild-type CMV strain whose antigens are not sufficiently similar to the test antigen, CMV antibodies may not be detected and the patient may be falsely misclassified as seronegative. Waner et al. have previously demonstrated that the use of different CMV strains as the sources of antigens may substantially alter the results of the CF assay (14). The influence of antigenic heterogeneity on other CMV antibody assays has not been scrutinized.

The unexpected observation of CMV CF seronegativity in three known congenitally CMV-infected infants and their mothers prompted an investigation that demonstrated antigenic heterogeneity as a cause of false CMV seronegativity in both the CF and IHA assays and suggested that the use of multiple CMV strains for producing antigens or antigen mixtures would reduce false seronegativity.

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MATERIALS AND METHODS

Patients. During a study of CMV infections among infants admitted to our intensive-care nursery, nine infants were identified as being congenitally infected, based on the recovery of CMV by standard viral culturing techniques from urine obtained at less than 5 days of age. None of the mothers and only one infant had symptomatic CMV disease. Cord and maternal sera were recovered from residual specimens submitted to clinical laboratories for other tests. CMV-specific CF antibody could not be detected on three separate occasions in cord sera or corresponding maternal sera in three of these infants and their mothers with a commercially prepared CMV CF antigen made from the AD169 strain (M. A. Bioproducts, Walkersville, Md.). Careful cross-standardization of the antigen, guinea pig complement, sheep erythrocytes, and anti-sheep erythrocyte antibody had been performed, and commercially obtained CMV CF-positive and -negative sera (M. A. Bioproducts) reacted appropriately. Cord and corresponding maternal sera from these nine congenitally infected infants and their mothers were used for subsequent testing. Cord sera were also randomly selected from healthy infants recently discharged from our normal nursery.

CF antigens. Glycine-extracted CF antigens were prepared locally from the AD169 and Davis strains (American Type Culture Collection, Rockville, Md.) and from viral isolates

from the three congenitally infected but initially CF-seronegative infants (A, B, and C) in a standard fashion (15). Confluent monolayers of locally derived human foreskin fibroblasts in triplicate 150-cm^2 plastic tissue culture flasks were infected with the appropriate viral strain. Fresh specimens of infected urine were used for A, B, and C. Resultant antigens were stored at −70°C. Antigens made from AD169 and Davis were titrated with antisera of a known titer for each antigen. Antigens made from the wild-type strains were titrated against commercial antisera with a known titer for AD169 (M. A. Bioproducts). An antigen mixture was also formulated with equal numbers of antigen units from AD169, Davis, and A preparations, so that upon microtiter testing, each well would receive 2 units of antigen, composed equally of AD169, Davis, and A antigens. Control antigens were prepared in the same manner, except that cell cultures were not infected with virus.

CF assay. CF antibodies to CMV were assayed by the previously described microtiter technique of Sever (11). The titer was expressed as the reciprocal of the highest serum dilution giving 75% fixation, as judged by the degree of hemolysis. A titer of ≥8 was considered seropositive. In each run, antigen controls, known positive and negative sera, reagent controls, and tests for serum anticomplementary activity were incorporated. Anti-sheep erythrocyte antibody, complement, and antigens were all titrated in a standard fashion (13). Individual serum specimens from congenitally infected infants and their mothers were assayed in parallel against all tested antigens simultaneously. Individual, randomly selected cord serum specimens were tested simultaneously with only locally prepared AD169, Davis, and A antigens.

IHA assay. A commercial test (Cetus Corporation, Emeryville, Calif.) was used to determine if sera were IHA seropositive or seronegative but not to determine absolute titers. Sera were diluted 1:4 with the supplied dilution buffer and then heat inactivated at 56°C for 30 min. Sera were then incubated for 180 min at room temperature with reconstituted, lyophilized, tanned, CMV AD169-sensitized human erythrocytes and nonsensitized control erythrocytes. Settling patterns in U-bottom microtiter wells were then compared with those produced by positive and negative control sera. Negative sera produced discrete, compact buttons with the sensitized cells, whereas positive sera produced a well-dispersed "mat appearance."

IgM-IIF assay. Antibodies to CMV in C, B, and their mothers were assayed by the IgM-specific CMV indirect immunofluorescent-antibody (IgM-IIF) assay as previously described (6), except that fluorescein-conjugated goat anti-human IgM antibody (Microbiological Research Corporation, Bountiful, Utah) was used as the conjugate instead of anti-human polyvalent immunoglobulin. The Davis strain of CMV was used for antigen production in accordance with the usual practice of the laboratory which performed the assay. Inadequate serum remained for IgM-IIF testing against antigens made from homologous virus.

RESULTS

Congenitally infected infants. Maternal and cord sera from all three infants and their mothers who were CMV positive but CF seronegative with commercial CMV AD169 antigen remained seronegative upon retesting with a commerical antigen and with a locally prepared glycine-extracted AD169 antigen. Maternal and cord sera were all seropositive when a glycine-extracted antigen made from the homologous viral isolates was used. All three mother-infant pairs were also seropositive when A antigen was used, but C and B antigens produced positive CF responses only when tested against sera from the corresponding infant and mother. Sera from C, B, and their mothers were seronegative when tested for IHA antibody with the provided AD169 antigen; no A serum remained for such testing (Table 1). No IgM-IIF was detected in C, B, or their mothers with the Davis antigen.

When the six congenitally infected infants who were CF seropositive with commercial AD169 antigen and their mothers were tested with a locally prepared glycine-extracted AD169 antigen, all remained seropositive. Upon testing with the other locally prepared antigens, four infant-mother pairs were positive for A, two were positive for C, and one was positive for B. All were seropositive when tested for IHA antibody.

Randomly selected cord sera. To further assess the impact of antigenic heterogeneity on CMV serologic testing, we tested 120 randomly selected cord sera for CF antibody with locally prepared glycine-extracted AD169, Davis, and A antigens (Table 2). Twelve sera were found to be anti-complementary and were excluded from further analysis. Among the remaining 108 sera, all three antigens produced similar results in 98 sera: 54 were seronegative, and 44 were seropositive (although CF titers varied fourfold or more in the same serum with different antigens in 18 sera). In the remaining 10 sera, there was evidence of antigen specificity: 4 were seropositive for A only, 3 were seropositive for A and Davis only, 2 were seropositive for AD169 and Davis only,

TABLE 1. Serologic results for nine congenitally CMV-infected infants and their mothers with antigens from several CMV strains[a]

Subject	CF titer with indicated antigen					IHA results with AD169 antigen[a]
	AD169C[b]	AD169L[c]	A	C	B	
A	—[d]	—	64	—	—	ND
Mother	—	—	32	—	—	ND
C	—	—	64	256	—	-
Mother	—	—	16	32	—	-
B	—	—	32	—	64	-
Mother	—	—	8	—	16	-
4	32	64	32	32	16	+
Mother	32	32	32	16	16	+
5	32	32	32	—	—	+
Mother	64	64	32	—	—	+
6	16	32	32	16	—	+
Mother	16	16	32	8	—	+
7	16	16	—	—	—	+
Mother	32	32	—	—	—	+
8	64	64	16	—	—	+
Mother	64	128	32	—	—	+
9	8	16	—	—	—	+
Mother	16	32	—	—	—	+

[a] +, Well-dispersed mat at well bottom; -, compact button at well bottom; ND, not determined.
[b] AD169C, Commercially prepared AD169 antigen.
[c] AD169L, Locally prepared AD169 antigen.
[d] —, Titer of <8.

TABLE 2. CMV serologic results for 120 randomly collected cord sera with glycine-extracted antigens from several CMV strains

No. of sera	CF titer[a] (range) with indicated antigen		
	AD169	Davis	A
54	–	–	–
44[b]	+ (8–128)	+ (8–256)	+ (8–128)
4	–	–	+ (8–64)
3	–	+ (8–32)	+ (16–32)
2	+ (8–16)	+ (16–32)	–
1	+ (16)	–	–
12	AC	AC	AC

[a] +, Titer of ≥8; –, titer of <8; AC, anticomplementary.
[b] In 18 sera, titers varied fourfold or more with different antigens.

and 1 was seropositive for AD169 only. When these 10 sera were tested against the antigen mixture made from AD169, Davis, and A all were seropositive.

When the 108 sera were tested in the commercial IHA test with AD169 antigen, the 98 sera with CF concordance for all three antigens were found to be IHA seronegative in 51 cases and IHA seropositive in 46 cases; nonspecific agglutination occurred in 1 case. Of the 10 sera that displayed antigen-specific seropositivity in the CF test, only 4 were seropositive in the IHA test, 3 that had been CF positive for AD169 only and 1 that had been CF positive for A and Davis only.

DISCUSSION

The use of CMV serology to facilitate the selection of blood products, organs for transplantation, and antenatal counselling makes the accuracy of serologic assays critically important. Misclassification of a patient as CMV seronegative could have great impact on patient management. The present investigation suggests that antigenic heterogeneity among CMV strains can result in such misclassification in both CF and IHA assays if only a single CMV strain is used as the antigen.

The antigenic heterogeneity of CMV has been previously noted. Weller et al. suggested this in 1960, based on serologic cross-neutralization assays (17). Waner et al. used antigens made from three different CMV strains in a CF assay and found that more than 20% of sera differed fourfold or more in titer with different strains and that 7% of sera were CF positive for only one of three antigens (14). Beutner et al. noted that CMV-specific lymphocyte blastogenesis was also dependent on which of three viral strains was used as the antigen (2). Cross-reactivity among CMV antigens is incomplete (20). This fact is emphasized by the three patients in the present report who were CF seronegative for commercial and locally prepared glycine-extracted AD169 antigens but strikingly CF seropositive when tested with antigens made from their own viral isolates. The present study suggests that the misleading effects of antigenic heterogeneity are not limited to the CF assay. The commercial IHA test, which uses AD169 antigen, failed to detect antibody in two congenitally infected infants who were strikingly CF seropositive when tested with their own antigen and failed to detect antibody in 6 of the 10 randomly selected cord sera that were CF seropositive for selected antigens. The IHA assay agreed completely with the CF assay for those subjects who were seropositive with all three antigens, except for two subjects who were CF negative but IHA positive.

The IHA test offers the advantages of IgM sensitivity, rapid turnaround times, and lack of interference by anticomplementary activity. A high incidence of nonspecific agglutination is reduced by the use of glutaraldehyde-fixed human type O erythrocytes and by careful attention to determining the optimal tannic acid concentration for each cell batch (18). The commercial IHA test insures a lower rate of nonspecific agglutination by incorporating both features. To absolutely confirm that antigenic heterogeneity is responsible for the IHA-seronegative but CF-seropositive wild-type sera in the present study would require performing the IHA assay with a battery of CMV antigens and demonstrating that those sera are IHA seropositive for the additional antigens. The many vagaries of the IHA assay that make a well-standardized commercial kit so attractive make the production of multiple, well-standardized tanned erythrocyte batches sensitized with different CMV antigens a challenge for a small laboratory. The observation of IHA seronegativity with AD169 antigen in two patients who were CF seronegative with AD169 antigen but CF seropositive with antigens made from their own CMV isolates is strongly suggestive of antigenic heterogeneity.

The survey of cord sera suggests that antigenic heterogeneity may not be a rare problem, since 7% (7 of 108) and 5% (5 of 108) would have been misclassified as CF seronegative if only AD169 or Davis antigen had been used, respectively. The IHA test with AD169 antigen failed to detect antibody in six of the sera that were CF seropositive when additional antigens were used. The observation of a fourfold or greater variation in the CF titer in the same serum with different antigens in 18 of the 44 sera that were seropositive for all three antigens further suggests antigenic heterogeneity. An antigen made by mixing three strains of virus successfully detected CF antibody in all 10 sera that showed antigenic specificity. An antigen mixture could obviate the need for separately testing sera against multiple CMV antigens. A mixture still may not include antigens that are sufficiently broadly reactive to detect antibodies against all CMV strains. The use of too many strains in such a mixture may decrease the concentration of some antigens so that inadequate antigen is present to detect antibodies specific for that antigen. The selection of an appropriate serum for titrating antigen strength and positive and negative control sera may be more challenging. In the present study, the strength of the antigens made from the wild-type strains was assessed with a commercial antiserum of a known titer against AD169; it was fortuitous that this serum cross-reacted with the wild-type strains. In future studies it would be preferable to assess antigen strength with serum from the subject from whom the virus strain was originally isolated. The use of a CMV antigen mixture made from only a few broadly reactive strains may diminish the risk of misclassifying a patient as seronegative because of antigenic heterogeneity.

It is possible that the observed discrepant antibody responses to antigens derived from CMV AD169 and endogenous virus represent the previously described high specifity of IgG antibodies formed early after a primary viral infection (5, 16). IgG antibodies formed later in the immune response are of a lower specificity and broadly cross-reactive with related viruses, as are IgM antibodies formed from a primary infection. Although IgM-IIF could not be detected in the available sera with antigen-specific CF antibody responses, the possibility of a recent primary infection cannot be rigorously excluded since (i) IgM responses do not invariably occur after a primary infection and (ii) although IgM antibodies following a primary infection are reportedly broadly reactive, it is possible that antigenic heterogeneity

between the endogenous CMV strains and the Davis strain used in the IgM-IIF assay may not have precluded detection. Unfortunately, insufficient serum was available for IgM retesting with additional CMV strains; since the CF seronegativity for AD169 antigen in the three congenitally infected infants and their mothers was unexpected, the need for larger quantities of sera for extensive retesting was not anticipated. Subsequent sera from these infants and their mothers were not available to determine whether more broadly reactive antibodies would develop with time. The inability to exclude the chronologic evolution of antibody specificity after a primary infection as a cause for the observed antigen-discrepant antibody responses does not decrease the relevance of our observations but further suggests the need to exercise caution when interpreting results of CMV serologic assays based on a single CMV antigen.

The present investigation does not suggest that CF is a better technique than IHA for the detection of CMV antibody. In addition to the advantages of IHA noted above, it should be noted that anticomplementary activity precluded CF testing in 10% of the test sera, whereas nonspecific agglutination precluded IHA testing in only 1%. It does appear that CMV antigenic heterogeneity may influence CMV IHA serology as well as CMV CF serology. Most previous studies comparing CMV serologic techniques have used the AD169 strain as the source of antigen for each technique or have not stated the CMV strain used (1, 3, 4, 8, 9); the effect of antigenic heterogeneity cannot be assessed in such circumstances. Few studies have correlated serologic results with virologic results. Fucillo et al. noted previously that IHA antibodies were not detectable in 2 of 20 patients with proven CMV infections (7). Further testing with other populations and other assay techniques will be necessary to determine the full impact of antigenic heterogeneity on the CMV serologic classification of individual patients.

Antigenic heterogeneity among community CMV strains may cause some sera to be misclassified as seronegative in the CF and IHA assays if only a single antigen is used. The use of multiple antigens or an antigenic mixture may decrease the incidence of such a misclassification. The possibility of false CMV seronegativity caused by antigenic heterogeneity should be considered when interpreting CMV serologic results.

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LITERATURE CITED

1. **Betts, R. F., S. D. George, B. B. Rundell, R. B. Freeman, and R. G. Douglas, Jr.** 1976. Comparative activity of immunofluorescent antibody and complement-fixing antibody in cytomegalovirus infection. J. Clin. Microbiol. **4**:151–156.
2. **Beutner, K. R., A. Morag, R. Deibel, B. Morag, D. Raiken, and P. L. Ogra.** 1978. Strain-specific local and systemic cell-mediated immune responses to cytomegalovirus in humans. Infect. Immun. **20**:82–87.
3. **Booth, J. C., G. Hannington, T. A. G. Aziz, and H. Stern.** 1979. Comparison of enzyme-linked immunosorbent assay technique and complement-fixation test for estimation of cytomegalovirus IgG antibody. J. Clin. Pathol. **32**:122–127.
4. **Brandt, J. A., J. D. Kettering, and J. E. Lewis.** 1984. Immunity to human cytomegalovirus measured and compared by complement fixation, indirect fluorescent-antibody, indirect hemagglutination, and enzyme-linked immunosorbent assays. J. Clin. Microbiol. **19**:147–152.
5. **Cremer, N. E., C. K. Cossen, G. R. Shell, and L. Pereira.** 1985. Antibody response to cytomegalovirus polypeptides captured by monoclonal antibodies on the solid phase in enzyme immunoassays. J. Clin. Microbiol. **21**:517–521.
6. **Faix, R. G., S. E. Zweig, J. F. Kummer, D. Moore, and D. J. Lang.** 1983. Cytomegalovirus-specific cell-mediated immunity in lower-socioeconomic-class adolescent women with local cytomegalovirus infections. J. Clin. Microbiol. **17**:582–587.
7. **Fuccillo, D. A., F. L. Moder, R. G. Traub, S. Hensen, and J. L. Sever.** 1971. Micro indirect hemagglutination test for cytomegalovirus. Appl. Microbiol. **21**:104–107.
8. **Griffiths, P. D., K. J. Buie, and R. B. Heath.** 1978. A comparison of complement fixation, indirect immunofluorescence for viral late antigens, and anti-complement immunofluorescence tests for the detection of cytomegalovirus specific serum antibodies. J. Clin. Pathol. **31**:827–831.
9. **Phipps, P. H., L. Gregoire, E. Rossier, and E. Perry.** 1983. Comparison of five methods of cytomegalovirus antibody screening of blood donors. J. Clin. Microbiol. **18**:1296–1300.
10. **Rubin, R. H., P. S. Russell, M. Levin, and C. Cohen.** 1979. Summary of a workshop on cytomegalovirus infections during organ transplantation. J. Infect. Dis. **139**:728–734.
11. **Sever, J. L.** 1962. Application of a microtechnique to viral serologic investigations. J. Immunol. **88**:320–329.
12. **Stagno, S., R. F. Pass, M. E. Dworsky, R. E. Henderson, E. G. Moore, P. D. Walton, and C. A. Alford.** 1982. Congenital cytomegalovirus infection: the relative importance of primary and recurrent maternal infection. N. Engl. J. Med. **306**:945–949.
13. **U.S. Public Health Service.** 1965. Standardized diagnostic complement fixation method and adaptation to microtest. U.S. Public Health Service publication no. 1228 (Public Health Monogr. 74). U.S. Government Printing Office, Washington, D.C.
14. **Waner, J. L., T. H. Weller, and S. V. Kevy.** 1973. Patterns of cytomegalovirus complement-fixing antibody activity: a longitudinal study of blood donors. J. Infect. Dis. **127**:538–543.
15. **Waner, J. L., T. H. Weller, and J. A. Stewart.** 1980. Cytomegalovirus, p. 622–627. *In* N. R. Rose and H. Friedman (ed.), Manual of clinical immunology, 2nd ed. American Society for Microbiology, Washington, D.C.
16. **Webster, R. G.** 1968. The immune response to influenza virus. III. Changes in the avidity and specificity of early IgM and IgG antibodies. Immunology **14**:39–52.
17. **Weller, T. H., J. B. Hanshaw, and D. E. Scott.** 1960. Serologic differentiation of viruses responsible for cytomegalic inclusion disease. Virology **12**:130–132.
18. **Yeager, A. S.** 1979. Improved indirect hemagglutination test for cytomegalovirus using human O erythrocytes in lysine. J. Clin. Microbiol. **10**:64–68.
19. **Yeager, A. S., F. C. Grumet, E. B. Hafleigh, A. A. Arvin, J. S. Bradley, and C. G. Prober.** 1981. Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. J. Pediatr. **98**:281–287.
20. **Zablotney, S. L., B. B. Wentworth, and E. R. Alexander.** 1978. Antigenic relatedness of 17 strains of human cytomegalovirus. Am. J. Epidemiol. **107**:336–343.