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Detection of Cytomegalovirus Antibody by Immune Adherence Hemagglutination (39588)

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Antibodies to cytomegalovirus (CMV) antigens can be detected by a variety of techniques including complement fixation (CF) (1-3), neutralization (4), indirect hemagglutination (5, 6) immunofluorescence (7), and platelet agglutination (8). Although heterogeneity exists among CMV antigens from different sources, the relatively broad reactivity and commercial availability of CMV antigens for CF as well as ease of performance have made CF the most routinely used serologic technique to detect antibody to CMV (anti-CMV). Recently tests to detect antigens and antibodies associated with hepatitis A and B viruses have been performed by immune adherence hemagglutination (IAHA), a complement-mediated assay more sensitive than CF (9-14). The present report describes the application of IAHA to the detection of anti-CMV in sera of multiply transfused patients.

Materials and methods. CMV antigen. CMV antigen of the AD 169 strain was purchased from Flow Laboratories (Rockville, Md). The antigen had been obtained by disruption of infected WI38 cells. Prior to use it was stored at −70°. Control antigen was prepared from disrupted, uninfected WI38 cells.

Serum samples. A pool of human serum known to have a CF titer of 1:32 was used as a positive anti-CMV control. For comparison of CF and IAHA, serum samples were obtained from 36 multiply transfused patients who had undergone open heart surgery (15). All samples were heated at 56° for 30 min to inactivate complement.

Buffers. Veronal-buffered saline (VBS) (pH 7.5) prepared as described by Kabat and Mayer (16) and supplemented with 0.1% bovine serum albumin (BSA) was used as diluent for antigen, antibody, and complement. A 40-m*M* EDTA solution in VBS-BSA was prepared as the diluent for human red blood cells and dithiothreitol (DTT).

Guinea pig complement. Guinea pig complement was obtained from a commercial source (Texas Biological Laboratories, Inc., Fort Worth, Texas) and diluted 1:75 for IAHA. Complement was prepared fresh in BSA-VBS diluent immediately prior to use.

Human red blood cells for IAHA. Type O, Rh-positive human red blood cells were obtained from normal volunteer blood donors. Because there is marked variation in red blood cell sensitivity as the indicator cell, red cells with the greatest sensitivity in IAHA were selected as the indicator cells in all subsequent tests. After collection, the red cells were suspended in 4 vol of Alsever's solution and preserved this way for 3 to 4 weeks at 4°. Beyond this period, the cells became progressively less sensitive as indicator erythrocytes. Prior to use, the red cells were washed once with EDTA-VBS-BSA buffer, then diluted in the same buffer to yield a 1% (vol/vol) suspension.

IAHA test to detect anti-CMV. Optimum concentrations of CMV antigen and antibody were determined by checkerboard titration of antigen against reference antiserum. In subsequent tests with the same lot of CMV antigen, the antigen was diluted in VBS-BSA to yield 4 IAHA units in 25 μl.

Duplicate sets of serial twofold dilutions were prepared in polystyrene U-bottom microtiter plates (Linbro Chemical Co., New Haven, Conn.) with 25-μl microdiluters (Cooke Engineering Co., Alexandria, Va.). Twenty-five microliters of diluted antigen was added to one set of serum dilutions and

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VBS–BSA buffer was added to the second. The plate was shaken briefly on a microtiter micromixer (Cooke Engineering Co.) and incubated in a humid chamber overnight at 4°. The following morning, 25 μl of freshly diluted guinea pig complement was added to each well, and the plate was briefly shaken and incubated for 40 min at 37°. Without delay, 25 μl of DTT (3 mg/ml) was added to each well followed by 25 μl of human red blood cell suspension. After thorough agitation on a micromixer, the plates were incubated at room temperature until negative hemagglutination patterns appeared in control wells, approximately 1 hr later. Hemagglutination was evaluated on a scale of zero to four, and patterns of three or four were considered positive, provided nonspecific hemagglutination was not observed in the corresponding control (buffer only) well. Immune adherence hemagglutination titers of anti-CMV were expressed as reciprocals of the highest serum dilution at which hemagglutination was observed.

A similar method was employed to determine IAHA titers of CMV antigen. Four IAHA units of anti-CMV serum in 25 μl of VBS–BSA was added to wells containing serial twofold dilutions of antigen.

CF test to detect anti-CMV. The same microtiter equipment and guinea pig complement were used to determine anti-CMV and CMV antigen titers by CF. For each well, 2 units of hemolysin and 1.8 to 2.0 units of complement were added according to the microtiter CF procedure described by Sever (17). Complement fixation titers of anti-CMV were expressed as reciprocals of the highest dilution at which 25% (3+) or less (4+) hemolysis was observed.

Results. Relative sensitivity. The relative sensitivity of IAHA and CF for the detection of CMV antigen and anti-CMV was determined in a checkerboard titration of a reference antiserum against CMV antigen (Fig. 1). For the detection of antigen, IAHA was 4- to 16-fold more sensitive than CF; IAHA was two- to eightfold more sensitive in the determination of anti-CMV. On the basis of this titration, CMV antigen was used at a dilution of 1:32 for IAHA and 1:8 for CF in subsequent assays of anti-CMV. At these dilutions there were 4 IAHA or CF units in 25 μl of antigen reagent. Neither

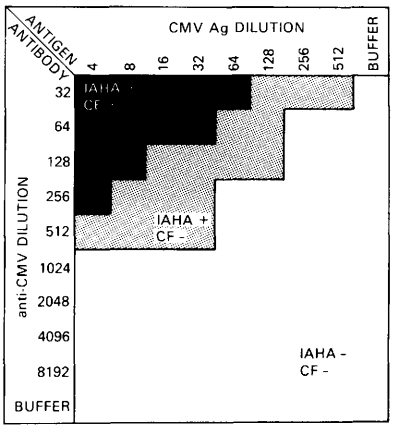

FIG. 1. Checkerboard titration of cytomegalovirus antigen (CMV Ag) against antibody to CMV (anti-CMV) by immune adherence hemagglutination (IAHA) and complement fixation (CF).

CF nor IAHA reactivity was observed when control antigen was used.

Anti-CMV in sera of transfused patients. Paired serum samples collected at least 1 month apart from multiply transfused patients were tested for anti-CMV by CF and IAHA (Table I). Sixteen of these 36 patients were selected on the basis of a seroresponse to CMV antigen as determined by CF in previous studies. Of 16 patients with at least fourfold anti-CMV rises measured by CF, 14 demonstrated at least four-fold rises by IAHA. Two patients (Numbers 15 and 16) with minimal rises in anti-CMV measured by CF showed no response by IAHA; both had preexisting antibody and developed no more than a two-fold convalescent antibody rise in the IAHA test. Although proportional increases of anti-CMV titer between pretransfusion and late samples were comparable between CF and IAHA, pretransfusion titers were slightly higher and convalescent titers were significantly higher when measured by IAHA for the 14 patients with anti-CMV rises by both techniques (Fig. 2). Sixteen patients (Numbers 21 through 36) who showed no rise in anti-CMV by CF showed no change in titer by IAHA; however, four patients (Numbers 17–20) had measurable rises by IAHA but no antibody response detected by CF. Seven patients had no detectable anti-CMV by CF in either sample but had detectable stable titers of anti-CMV by IAHA in both (Num-

TABLE I. ANTI-CMV[a] IN PAIRED SERA OF MULTIPLY TRANSFUSED PATIENTS.

Patient	Date[b]	Anti-CMV inverse titer		Patient	Date[b]	Anti-CMV inverse titer	
		CF[c]	IAHA[d]			CF[c]	IAHA[d]
1	4 Weeks	2	64	19	Pre	64	256
	8 Weeks	128	2048		22 Weeks	<256[e]	1024
2	Pre	8	<8	20	Pre	16	256
	9 Weeks	64	256		11 Weeks	<64[e]	≥2048
3	Pre	32	256	21	Pre	16	128
	14 Weeks	128	1024		8 Months	32	128
4	Pre	4	8	22	Pre	32	64
	24 Weeks	64	2048		24 Weeks	32	64
5	4 Weeks	<2	<8	23	4 Weeks	32	64
	11 Months	512	4096		12 Weeks	16	64
6	Pre	32	256	24	Pre	32	128
	23 Weeks	128	1024		10 Weeks	<128[e]	128
7	Pre	<2	<8	25	Pre	<2	<8
	19 Weeks	32	128		8 Months	<8	<8
8	Pre	<2	<8	26	Pre	<4	<8
	17 Weeks	32	32		7 Months	<8	<8
9	Pre	<2	8	27	Pre	64	512
	22 Weeks	16	32		26 Weeks	<256[e]	1024
10	Pre	<2	16	28	Pre	8	64
	10 Weeks	64	512		25 Weeks	<32[e]	64
11	2 Weeks	<2	32	29	Pre	8	128
	13 Weeks	64	256		8 Months	<32[e]	64
12	Pre	<2	<8	30	2 Weeks	<2	8
	10 Weeks	64	256		12 Weeks	<2	16
13	Pre	<2	8	31	Pre	<2	32
	20 Weeks	64	128		24 Weeks	<8	32
14	4 Weeks	<2	32	32	4 Weeks	<8	256
	10 Weeks	128	512		10 Months	<8	512
15	Pre	4	64	33	4 Weeks	<16	32
	24 Weeks	32	128		7 Months	<16	32
16	Pre	<2	8	34	Pre	<4	128
	17 Weeks	32	16		26 Weeks	<4	64
17	Pre	<2	<8	35	Pre	<2	64
	24 Weeks	<8	64		26 Weeks	<8	64
18	Pre	<2	<8	36	10 Weeks	<8	512
	23 Weeks	<8	512		12 Months	<8	256

[a] Antibody to cytomegalovirus antigen.
[b] Relative to transfusion.
[c] Complement fixation.
[d] Immune adherence hemagglutination.
[e] These sera were tested beginning at a dilution fourfold greater than the titer of the pre-transfusion sample.

bers 30–36). The Kendal coefficient of association (18) between anti-CMV rises detected by CF and IAHA was 0.671 ($P < 0.001$) (Table II). In tests of reproducibility, no two determinations varied by more than one dilution when IAHA testing was repeated on the same serum sample.

Kinetics of anti-CMV development. The temporal relationship between the development of CF and IAHA antibody to CMV antigen was evaluated in serial serum samples obtained from two of the individuals evaluated above. Patient No. 4 had minimal pretransfusion anti-CMV, which remained unchanged for 7 weeks as determined by CF. Anti-CMV detected by IAHA began to increase 2 weeks after transfusion. After 7 weeks, CF and IAHA antibody titers rose in parallel fashion, but IAHA titers remained higher than CF titers (Fig. 3a). Pretransfusion anti-CMV was not detected by either test in serum from patient No. 5. Between 6 and 16 weeks after transfusion, anti-CMV developed and was detected by both IAHA and CF. For each sample, the IAHA titer was slightly higher than the CF titer, but the patterns of changing titer were similar (Fig. 3b).

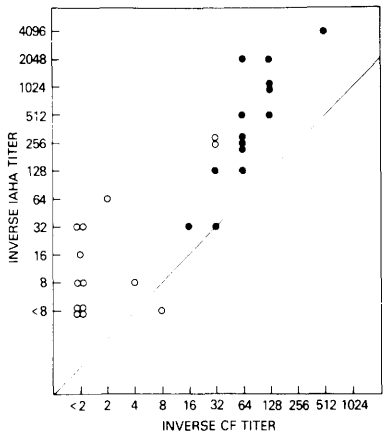

FIG. 2. Immune adherence hemagglutination (IAHA) titers expressed as reciprocal of dilution (given as inverse titers) plotted as a function of complement fixation (CF) titers of pretransfusion sera (○) and post-transfusion sera (●) for 14 patients with rises in titer of antibody to cytomegalovirus. Points falling above the diagonal line represent enhanced sensitivity of IAHA compared to CF.

Discussion. In immune adherence hemagglutination, there is complement-mediated agglutination of indicator erythrocytes rather than the hemolysis observed in complement fixation tests. Surface receptor sites on nonsensitized red blood cells adhere to the third component of complement (C_3) which is bound to an antigen–antibody complex and complement components C_1, C_4, and C_2. Dithiothreitol is added to protect C_3 from C_3-inactivator and, thus, to stabilize the hemagglutination pattern (9). The term "immune adherence" was coined by Nelson (19) to describe a phenomenon recognized half a century earlier (20, 21). To Nishioka (22) goes the credit for characterizing complement component participation in immune adherence and popularizing IAHA as a sensitive technique for the detection of bacterial and viral antigens. Subsequent work by Ito and Tagaya (23) established the usefulness of IAHA for detecting a variety of viral antigens and antibodies and demonstrated a 10- to 100-fold increase in sensitivity over complement fixation tests. Since 1971, IAHA has been used extensively in Japan to detect hepatitis B surface antigen and antibody. With sensitivity 40 to 100 times greater than the complement fixation test and comparable to radioimmunoassay techniques, IAHA is used routinely to screen blood donors and for epidemiologic investigations (9, 10). Most recently, this sensitive assay has been used to detect antigens and antibodies associated with hepatitis A virus (11, 12, 14) and hepatitis B core antigen and antibody (13).

Cytomegalovirus antigen used in this study was obtained from the supernatant of pelleted, disrupted cells in which CMV was grown. Despite the lack of additional purification, the antigen preparation was relatively clean and easily usable in the test. Still, several discrepancies occurred between IAHA and CF results. In two cases of fourfold titer increases detected by CF, only twofold rises were detected by IAHA. For these two, pretransfusion antibody, undetected by CF, was observed by IAHA. In addition, cases 30–36 had no detectable anti-CMV by CF, but anti-CMV was demonstrated by IAHA in both specimens, and IAHA detected four significant serologic responses which were missed by CF. A factor which further compounds the difficulty of assessing these serologic events is the heterogeneity of CMV antigens and the incomplete cross-reactivity between them (24). Some of the discrepancies noted in this study may result from differing degrees of cross-reactivity among CMV strains as measured by CF and IAHA, since only the AD 169 strain was employed as antigen in this study. In general, however, the parallelism between CF and IAHA in all cases of convincing sero-response and the enhanced sensitivity of IAHA make IAHA an attractive alternative to CF. Moreover, the increased sensitivity of IAHA reveals that a greater proportion of anti-CMV responses are reinfection rather than primary exposures. Although other CMV strains were not available for testing, their inclusion in

TABLE II. CONCORDANCE BETWEEN RISES IN ANTI-CMV[a] TITERS DETECTED BY CF[b] AND IAHA.[c]

	IAHA	
CF	Rise	No rise
Rise	14	2
No rise	4	16

[a] Antibody to cytomegalovirus.
[b] Complement fixation.
[c] Immune adherence hemagglutination.

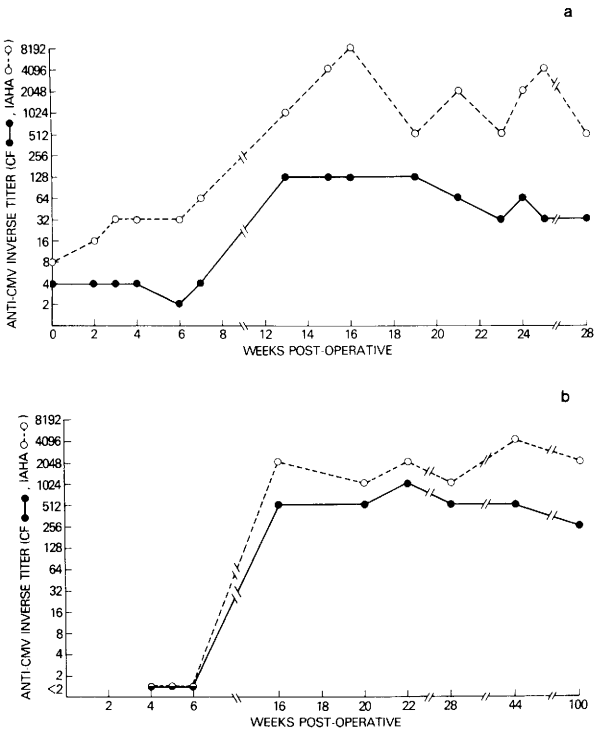

FIG. 3. Serial determinations of antibody to cytomegalovirus (anti-CMV) by complement fixation (CF) and immune adherence hemagglutination (IAHA) after transfusion in the sera of (a) a patient with pretransfusion anti-CMV and (b) a patient with no pretransfusion anti-CMV.

the preparation of a multivalent CMV antigen would make this test even more valuable.

In addition to enhanced sensitivity, IAHA required one-quarter of the CMV antigen reagent required for CF. Furthermore, since similar IAHA tests are available to detect antibody to hepatitis B surface (9) and core (13) antigens and hepatitis A antigen (11, 12, 14), theoretically, replicate dilutions of a single heat-inactivated serum sample could be tested for antibody to hepatitis and CMV antigens with the same technique in one microtiter plate. Since these viruses may be transmitted by transfusion, determination of these antibody responses is important to determine the etiology of transfusion-associated hepatitis and other syndromes. Perhaps, too, an IAHA test for antibody to Epstein–Barr virus, another agent transmitted by transfusion, can be developed, making serologic testing of post-transfusion patients even more efficient.

The only IAHA reagents difficult to obtain, the indicator red blood cells, can be found by screening cells from many blood donors with blood type O. Once suitable donors are identified, their erythrocytes may be stored in four parts of Alsever's solution (vol/vol) for up to 1 month for use in IAHA. Unlike previously reported IAHA assays (9), no prozone phenomenon was observed in the application of IAHA to CMV antigen–antibody detection.

Finally, no major difference was noted in the kinetics of anti-CMV development between CF and IAHA in the two patients studied. In contrast to the earlier development of CF antibody than IAHA antibody to hepatitis A antigen (11, 12), significant rises in anti-CMV occurred at the same time, when measured by CF and IAHA.

Summary. The principle of complement-mediated immune adherence was applied to development of an immune adherence hemagglutination (IAHA) assay for antibody to cytomegalovirus (anti-CMV). Compared to complement fixation (CF), IAHA was

two to eight times more sensitive for the detection of anti-CMV. In paired serum samples from multiply transfused patients, the concordance between rises in anti-CMV titers detected by CF and IAHA was 0.671 ($P < 0.001$). Convalescent titers measured by IAHA were significantly higher than those determined by CF in serum pairs in which anti-CMV rises were demonstrated by both techniques, and IAHA more commonly revealed anti-CMV in pretransfusion serum samples. No difference was noted between the kinetics of CF and IAHA antibody development after transfusion. Immune adherence hemagglutination, we conclude, is an efficient, sensitive alternative to CF for the detection of anti-CMV.

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