Improved Indirect Hemagglutination Test for Cytomegalovirus Using Human O Erythrocytes in Lysine

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A modified indirect hemagglutination test for cytomegalovirus antibodies is described in which glutaraldehyde-fixed human O cells, rather than sheep cells, are used. Nonspecific hemagglutination was reduced by use of the optimal tannic acid concentration for each cell batch and the addition of 0.1 M lysine to the phosphate-buffered saline in which the fixed, tanned, sensitized cells were resuspended for use in the test. Three of 349 sera showed nonspecific hemagglutination by this technique. Antigen made by freezing phosphate-buffered saline (pH 7.2) over an infected monolayer can be used at dilutions of 1:8 to 1:15. Fixed, tanned, sensitized cells ready for use in the test can be stored in liquid nitrogen for up to 8 months. Use of cryoprotectants and washing after thawing is unnecessary. Simplification of the assay permits one person to screen 300 sera in 1 day or to determine the immune status of a potential donor or recipient in 45 min after the test is set up. The modified assay compares favorably in sensitivity with the complement fixation test and with previously described methods for performing the indirect hemagglutination test.

Screening of blood and organ donors for immune status to cytomegalovirus (CMV) may be a useful means of preventing the spread of infection to certain types of patients whose risk of serious disease is high. Transmission of CMV from seropositive donors to seronegative recipients of renal transplants has been associated with fever, hepatitis, pneumonia, and an increase in rejection of transplanted kidneys (2, 6). Transfusion-acquired CMV infections can cause serious illness in recipients of marrow transplants and in premature infants (8, 10, 11, 15). In addition, premature infants who acquire CMV infections postnatally are potentially subject to the long-term sequelae associated with intrauterine CMV infections (5, 9, 12).

The indirect fluorescent antibody test has been found to be of sufficient sensitivity to predict potential CMV transmission by organ donors (3). The anti-complement indirect immunofluorescent test and the indirect hemagglutination test (IHA) have been shown to be comparable in sensitivity (11). However, the IHA is more practical for use in screening large numbers of donors, as would be necessary to select blood products for marrow transplant recipients and premature infants. The IHA is more sensitive than the complement fixation test (1, 4) and, in addition, measures immunoglobulin M antibody more reliably (4). The detection of immunoglob-

ulin M antibody is important because persons who have been recently infected can reasonably be expected to transmit CMV at a high rate. The seroconversion rate among blood donors is at least 0.8% a year (unpublished data) and is estimated to be 1 to 2% in the general population (13).

Despite the sensitivity and suitability of IHA for mass screening, nonspecific hemagglutination occurs in 5 to 20% of sera if the IHA technique is performed as originally described (1). The purpose of this paper is to describe modifications of the technique which eliminate nonspecific hemagglutination, to emphasize those variables upon which the sensitivity of the assay is dependent, and to describe the conditions under which glutaraldehyde-fixed, tanned, sensitized cells can be stored for long periods.

METHODS

Glutaraldehyde fixation of erythrocytes. Human whole blood, freshly drawn from a donor with type O erythrocytes, was mixed with 3.8% sodium citrate in a ratio of 1 part of whole blood to 1.2 parts of sodium citrate and held at 4°C for up to 5 days until use. The erythrocytes were washed three times with cold, 0.15 M phosphate-buffered saline (PBS) (pH 7.2) and were added slowly to a 1% solution of glutaraldehyde (EM grade, Polysciences, Inc.) in 0.15 M PBS (pH 8.2) so that the final cell concentration was 1%. The suspended cells were gently agitated at 4°C for 30

min. After removal of the glutaraldehyde solution by centrifugation at 900 × g for 15 min, the cell pellet was washed five times with PBS (pH 7.2). The cells were resuspended in 0.15 M PBS (pH 7.2) to a concentration of 5% and stored at −70°C for periods up to 6 months.

Preparation of CMV antigen for the IHA test. Freshly sheeted monolayers of human foreskin fibroblasts were infected with the stock strain of CMV, AD 169, which had been passed six times after removal from storage at −70°C. When the cytopathic effect was advanced, the infected cells were removed by trypsinization, and the cells from 6 to 10 tubes (approximately 2 × 10^5 cells per tube) were used to inoculate two roller bottles (108 by 104 mm) containing freshly sheeted confluent monolayers of human foreskin fibroblasts at cell passage level 13 or less. After absorption for 1 h at 37°C, Eagle minimal essential medium with 2% fetal calf serum and penicillin, gentamicin, and amphotericin was added. The medium was replaced after 18 h. Within 4 to 5 days, 100% of the cells showed cytopathic effect. The medium and trypsinized cells from the two bottles were combined and used to inoculate 20 to 26 plastic roller bottles (108 by 104 mm) in the same manner as described above. Cytopathic effect was visible at 24 h and involved 100% of the cells by day 4 after inoculation. At 5 to 7 days after inoculation, the medium was removed, and the infected monolayers were each washed twice with 0.85% sodium chloride. Then 20 to 22 ml of 0.15 M PBS (pH 7.2) was added to a single bottle and frozen over the monolayer while the bottle was slowly rotated in a bath of alcohol and dry ice. After thawing at room temperature, the same 20 to 22 ml of PBS was frozen over the monolayers of two or three additional bottles so that the total amount of PBS used was 5 to 7.5 ml per roller bottle. The PBS containing the antigen was clarified by centrifugation at 900 × g for 20 min, dispensed, and stored at −70°C. Storage for up to 18 months did not result in loss of potency. Control antigen was prepared in the same manner from uninfected monolayers.

The optimal dilution of antigen was determined by block titration against a known positive serum pool using cells treated with the optimal tannic acid concentration for that cell batch (see below) and varying dilutions of antigen. The optimal dilution of antigen varied between 1:8 and 1:15.

All assays reported here were performed with antigens made as described above; however, equivalent results were also obtained using antigens prepared by sonic treatment of infected cells in 0.15 M PBS (pH 7.2) or citrated water. After clarification by centrifugation, the supernatant fluid containing the antigen could be stored as above.

Preparation of tanned erythrocytes and sensitization with CMV antigen. Sterile reagents were used because contaminated PBS causes nonspecific hemagglutination. Erythrocytes that had been freshly collected in sodium citrate, or frozen glutaraldehyde-fixed cells that had been removed from storage at −70°C and thawed at 45°C, were washed three times in 0.15 M PBS (pH 7.2). The optimal tannic acid concentration was determined for each cell batch by testing known positive and negative sera against cells treated 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. Tannic acid dilutions were prepared immediately before use. Since the avidity of tannic acid for glass or plastic may influence its availability, vessels of the same material were used in parallel manner in specific steps of the tannic acid and antigen titrations and in the IHA assays.

The washed cells were resuspended to 2.5% (vol/vol) in 0.15 M PBS (pH 7.2) and incubated with an equal volume of the optimal tannic acid concentration for the cell batch at 37°C for 10 min. The cells were pelleted, washed once in 0.15 M PBS (pH 7.2), and resuspended to 2.5% (vol/vol) in 0.15 M PBS (pH 6.7). The cells were then divided into two portions. One portion was sensitized by incubation with an equal volume of the optimal dilution of antigen in 0.15 M PBS (pH 6.7) for 30 min at room temperature with gentle agitation every 10 min. The other portion, which was to serve as the cell control, was incubated in like manner with a 1:8 dilution of control antigen or in PBS (pH 6.7) alone. The cells were pelleted and washed three times in 0.15 M PBS (pH 7.2) containing 1% normal rabbit serum. The tanned sensitized cells and the control tanned cells were resuspended in 0.15 M PBS (pH 7.2) containing 1% normal rabbit serum and 0.1 M lysine and were used at a concentration of 1% (vol/vol) in the test.

Freezing sensitized cells. Glutaraldehyde-fixed, tanned, sensitized cells and control cells were frozen in liquid nitrogen or at −70°C at concentrations of 1% or 10% in 0.15 M PBS (pH 7.2) containing 1% normal rabbit serum and 0.1 M lysine. Cells were frozen without use of a cryoprotectant and were placed in liquid nitrogen while at room temperature. Cells stored at −70°C were quick-frozen in dry ice and alcohol before being placed at −70°C. Cells were thawed at room temperature.

IHA test. Sera were diluted to 1:8 in 0.15 M PBS (pH 7.2) containing 1% normal rabbit serum and 0.1 M lysine and inactivated at 56°C for 30 min. After 0.1 ml of heat-inactivated serum had been placed in the first well of each row of microtiter "V" plates (Cooke), twofold serial dilutions were made in 0.05 ml of PBS (pH 7.2) containing 1% normal rabbit serum and 0.1 M lysine with calibrated microtiter loops. Sensitized and control cells were added to the appropriate wells in a volume of 0.025 ml. Three known positive sera with different titers and one known negative serum were run with each assay. Controls for nonspecific hemagglutination included tanned cells alone, tanned-sensitized cells alone, and tanned cells with a 1:8 dilution of each serum. Plates were agitated on a Microshaker II (Dynatech) at setting 5 for 15 s. The plates were then placed on a surface free from vibration. The cells were allowed to settle for 45 min. The titer was considered to be the highest dilution of serum that caused 3 to 4+ agglutination.

When the amounts of tannic acid and antigen used were optimal, the positive serum pool had a titer of 1:512 ± 1 dilution. The complement fixation titer of this pool was 1:64 ± 1 dilution.

RESULTS

The choice of the optimal tannic acid concentration is critical in determining the sensitivity

of the assay as shown in Table 1. Use of too concentrated a solution of tannic acid results in nonspecific hemagglutination. Too dilute a solution results in losses in titer of fourfold or more. Use of an optimal dilution of antigen is equally important in assuring sensitivity of the assays (Table 2).

Use of fresh human O cells instead of sheep cells eliminated the need for absorbing the sera with the cells. The geometric mean titer of 70 seropositive sera was 1:179 when fresh human O cells were used, as compared to 1:76 when glutaraldehyde-fixed sheep cells were used. Although fresh cells were sensitive, it proved to be impossible to predict the optimal tannic acid requirement when unfixed cells were stored in sodium citrate at 4°C. Multiple tannic acid titrations on the cells of a single donor are recorded in Fig. 1. The filled circles represent assays considered satisfactory (titer ≥ 1:512), and the open circles represent assays giving a suboptimal titer. Cells were always titered against four tannic acid concentrations, but concentrations giving nonspecific hemagglutination are not recorded in the figure. The lines connect sequential tests on the same cell batch. Of three batches tested at 7 to 9 days, two gave optimal results at a tannic acid concentration of 1:160,000 and one gave an unsatisfactory result at this concentration of tannic acid. The tannic acid requirement stabi-
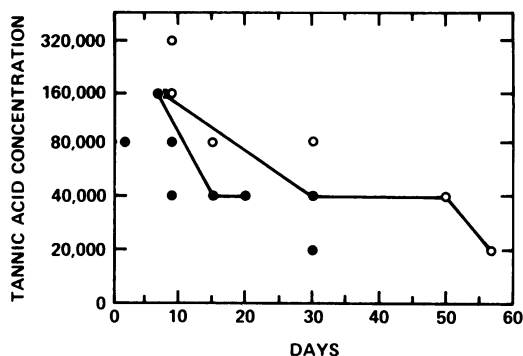

FIG. 1. Tannic acid titrations on human O cells from a single donor after various periods of storage in sodium citrate at 4°C. Tannic acid concentrations are shown at which sensitivity was (●) optimal and (○) suboptimal. The connecting lines link retitrations on the same batch of cells.

lized as the cells aged at 4°C, but the cells also lost sensitivity. The necessity of repeating the tannic acid titration before each IHA test made the use of fresh cells impractical.

Glutaraldehyde-fixed O cells showed as much nonspecific hemagglutination as glutaraldehyde-fixed sheep cells. Since the nonspecific hemagglutination could be caused by a reaction between glutaraldehyde and the plastic bottom of the microtiter well, fixed and tanned cells were suspended in various concentrations of lysine. It was determined that 0.1 M lysine eliminated this nonspecific reaction. Lysine also decreased the settling time so that the test could be read 45 to 75 min after completion. It was necessary to add the lysine to the PBS on the day the test was performed because older solutions resulted in indistinct patterns.

The sensitivity of the glutaraldehyde-fixed O cells in lysine compared favorably with the glutaraldehyde-fixed sheep cells in the standard assay (Fig. 2). The geometric mean titer of the 47 seropositive sera was 1:76 using the sheep cells and 1:199 using the O cells. The apparent increase in sensitivity may, in part, be due to increased confidence in reading the endpoint due to the elimination of nonspecific hemagglutination. Nonspecific hemagglutination detected by the tanned cell control occurred in 3 of 349 (0.9%) sera. None of 215 sera tested with tanned cells coated with control antigen showed nonspecific hemagglutination attributable to the control antigen.

Glutaraldehyde-fixed, tanned sensitized cells can be stored at concentrations of 1 or 10% in liquid nitrogen for a period of at least 8 months. Use of cryoprotectants and careful control of freezing rate are unnecessary. Table 3 shows

TABLE 1. Dependency of IHA assay on optimal tannic acid concentration

Optimal antigen + tannic acid concn	Reciprocal of CMV IHA titer			
	Serum H	Serum L	Serum DC	Serum N
1:40,000	NSH[a]	NSH	NSH	NSH
1:80,000	NSH	NSH	NSH	NSH
1:160,000	≥1,024	≥1,024	128	<8
1:320,000	256	128	±8	<8

[a] NSH, Nonspecific hemagglutination.

TABLE 2. Dependency of IHA on optimal concentration of antigen

Serum	Reciprocal of CMV IHA titer	
	Optimal tannic acid + optimal antigen	Optimal tannic acid + 50% of optimal antigen
P278	≥1,024	64
J931	512	32
B290	512	32
R184	256	64
HPP	≥256	128
LPP	≥256	64
M269	64–128	16
J384	16	±8
N1	<8	<8
N2	<8	<8

retitrations performed on 1% cells, which were thawed and used without further processing after periods of storage of 1 to 29 days. Figure 3 shows the concordance between the original titers and the titers obtained using the same 1% fixed, tanned sensitized cells that had been stored in liquid nitrogen for periods of 4 and 6 to 8 months.

Fixed, tanned, sensitized cells that have been quick-frozen in dry ice and alcohol can also be stored at −70°C for up to 1 month. Longer periods of storage at −70°C result in indistinct patterns in some cell batches.

DISCUSSION

Recent studies have confirmed that the CMV IHA is comparable in sensitivity to the CMV indirect fluorescent antibody test (4, 7). The modifications described make it practical to screen blood donors routinely for antibody to CMV as they presently are for hepatitis B antigen. The purpose of such screening would be to eliminate the use of blood products from donors

FIG. 3. IHA titers obtained using freshly sensitized, glutaraldehyde-fixed human O cells as compared to titers obtained using the same cells after storage in liquid nitrogen for 4 months or 6 to 8 months.

who have experienced a CMV infection in certain patient populations. Present data suggest that this may prove to be a useful means of decreasing transfusion- and transplant-associated CMV infections (3; A. S. Yeager, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, no. 512, 1978).

The use of human O cells instead of sheep cells eliminates the necessity of absorbing the sera with cells. Fixation of cells in 1% glutaraldehyde permits long-term storage of fixed cells at −70°C so that the optimal dilution of tannic acid for each cell batch may be determined. The use of the proper tannic acid concentration eliminates nonspecific hemagglutination and is critical in establishing sensitivity of the assay. The addition of 0.1 M lysine to the PBS in which the sera are diluted and to the PBS used to dilute the cells to the final working concentration eliminates nonspecific hemagglutination and decreases the time necessary for the cells to settle. It is probable that the lysine is blocking residual free aldehyde groups (14). Nonspecific hemagglutination as detected by the tanned cell control occurred in 0.9% of the sera tested in this study but may be higher in laboratories that receive sera which have become contaminated in transit. None of 215 sera from normal adults showed nonspecific hemagglutination to the tanned-cell-control-antigen complex unless they also showed such a reaction to tanned cells alone. Tanned cells alone, therefore, identify all false-positive reactions in normal adults and can be used at much lower costs than cells coated with control antigen. In testing patient populations
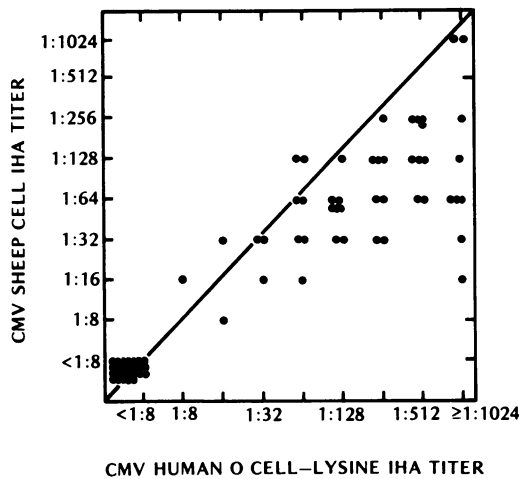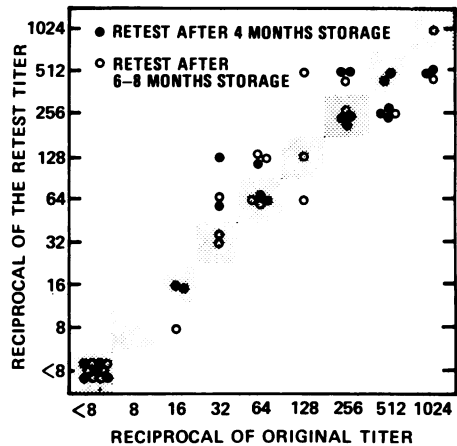
FIG. 2. Sensitivity of the IHA assay using glutaraldehyde-fixed sheep cells compared to glutaraldehyde-fixed human O cells in lysine.

TABLE 3. IHA titers using freshly sensitized cells compared to titers using the same cells after various periods of storage in liquid nitrogen

Serum	Reciprocal of titer				
	Fresh cells	Period of cell storage:			
		1 day	7 days	13 days	29 days
HPP	256–512	256–512	512	256–512	256–512
LPP	256–512	256	256	128	256
DC	32	16–32	32	16	16–32
N1	<8	<8	<8	<8	<8

with a high incidence of autoantibodies, use of a control consisting of a tanned-cell–control-antigen complex might prove necessary.

Fixed, tanned sensitized cells can be stored in liquid nitrogen for periods of up to 8 months and at −70°C for periods of up to 1 month. The use of cryoprotectants, careful control of freezing and thawing rates, and washing after thawing are not necessary.

Using freshly prepared cells, one technician can screen 300 sera for antibody in 1 day. Using pre-prepared cells, the antibody status of a donor can be determined in 45 min after the test is set up. The method compares favorably in sensitivity with the complement fixation assay and with the IHA using glutaraldehyde-fixed sheep cells.

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