Freeze-Dried Erythrocytes for an Indirect Hemagglutination Test for Detection of Cytomegalovirus Antibodies

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An indirect hemagglutination test with lyophilized, fixed, tanned, and cytomegalovirus (CMV)-sensitized sheep erythrocytes for the detection of CMV antibodies is reported. To avoid nonspecific hemagglutination, cells were fixed with glutaraldehyde or Formalin directly in whole blood. The lyophilized, CMV-sensitized erythrocytes obtained by this technique were stable up to 9 months at 37°C and retained the same reactivity as fresh, CMV-sensitized cells. Indirect hemagglutination performed with lyophilized, sensitized cells was highly efficient in detecting CMV-antibodies as compared with complement fixation and enzyme immunoassay.

The multiple advantages of the indirect hemagglutination technique (IHA) for detection of human cytomegalovirus (CMV) antibodies as compared with other serological methods have been reviewed recently (4). IHA, however, poses problems as a routine test with regard to the preparation of the reagents and, especially, the sensitizing of erythrocytes (RBC) with virus. Another major drawback in the standardization of IHA is the instability of sensitized RBC.

Yeager (6) developed an improved technique for IHA by using human O RBC fixed in glutaraldehyde and frozen in liquid nitrogen.

We tried to freeze-dry fixed, CMV-sensitized sheep RBC, and noticed that the main obstacle to the use of such RBC in IHA was a nonspecific agglutination occurring after lyophilization of RBC.

We have been able to demonstrate that the origin of this nonspecific agglutination was the aldehyde fixation of centrifuged and washed RBC. If the RBC were fixed by glutaraldehyde or formalin in the whole blood before centrifugation, they could then be sensitized and lyophilized without introducing nonspecific agglutination. Such lyophilized, sensitized RBC proved stable for long periods of storage, even at 37°C.

This paper describes in detail the procedure for the preparation of lyophilized, CMV-sensitized RBC and gives results obtained with such RBC for quantitative detection of CMV antibodies in human sera.

MATERIALS AND METHODS

Selection of an RBC donor sheep. In IHA an important variable is the quality of the RBC from different animals. We therefore selected a good donor from six young sheep. The donor animal was chosen according to the results of a comparative CMV-IHA test performed with RBC of the six sheep, prepared as described below.

Processing and fixation of RBC. Whole blood was drawn from the donor sheep into a bottle containing Alsever solution (AS) (20.5 g of glucose, 0.2 g of NaCl, 8 g of trisodium-citrate, 0.55 g of citric acid, in a total of 1 liter of distilled water). Blood and AS were mixed in equal volumes. The mixture of blood and AS was brought immediately to the laboratory, and the fixation of RBC was carried out the same day.

We used a 70% solution of electron microscope grade glutaraldehyde (Janning, Paris, France). For long storage, glutaraldehyde was diluted in distilled water to 10% concentration and distributed in 1-ml vials that were sealed and kept at 4°C. A 1:150 or 1:250 final dilution was made in phosphate-buffered saline (PBS) (pH 7) just before use and mixed with sheep blood in AS in equal volumes. The mixture was incubated overnight at 37°C with gentle stirring. The next day, fixed RBC were sedimented at $1,000 \times g$ for 20 min and washed five times in PBS.

For Formalin fixation, the commercial 30% solution (Prolabo) was diluted to obtain a 4% solution in trisodium citrate (0.15 M). Equal parts of 4% formalin and sheep blood in AS were mixed and processed as above.

Preparation of CMV antigen. Human embryo lung fibroblast (HEL) cells strain 809 grown in Eagle minimum essential medium supplemented with 10% newborn calf serum were used throughout this study.

Confluent cultures of HEL in 75-cm² plastic flasks

were inoculated with 0.5 ml of AD-169 human CMV stock suspension, and 15 ml of serum-free minimum essential medium was added. Four days later, when an extensive cytopathic effect had developed, infected cells were trypsinized and then inoculated into four other HEL cultures in 75-cm² flasks, and 15 ml of serum-free medium was added.

After another 4 to 5 days, when an advanced cytopathic effect was observed, all cells were trypsinized and passaged onto four Roux plastic bottles (150 cm²) containing confluent monolayers of HEL, and 50 ml of serum free minimum essential medium was added.

After 4 to 5 days the medium was discarded, and all the cells from the four Roux flasks were scraped and suspended in 8 ml of PBS (pH 7). The infected cell suspension was then frozen and thawed three times and centrifuged at $1,000 \times g$ for 20 min. After centrifugation, the supernatant fluid, considered as CMV antigen, was distributed in 0.5-ml volumes in vials and stored in liquid nitrogen.

The appropriate dilution of the antigen used to sensitize RBC was determined in an IHA test as described below. The optimal dilution varied between 1:15 and 1:60.

Preparation of tanned RBC. A 3% suspension (in PBS, pH 7) of fresh unfixed, or glutaraldehyde- or Formalin-fixed RBC was mixed in equal volumes with a tannic acid solution (in PBS) and allowed to stand for 15 min at 37°C.

The concentration of tannic acid solution varied (1:40,000 to 1:160,000) and had to be determined for each donor sheep and for each batch of viral antigen, as described previously (1).

Sensitization of tanned RBC with CMV antigen. For sensitization, tanned RBC were centrifuged and suspended at a concentration of 3% in 0.15 M PBS (pH 6.8).

Equal volumes of tanned RBC and CMV antigen diluted in PBS were mixed and incubated for 15 min at room temperature.

The CMV-sensitized RBC (CMV-RBC) were washed once in PBS and resuspended to 6% in sterile PBS containing 0.2% crystallized bovine serum albumin. Lactose (5%) was also added to CMV-RBC before lyophilization.

Lyophilization procedure. The fixed CMV-RBC suspension was distributed in 0.5-ml volumes into 5-ml lyophilization vials, stirred gently, and frozen immediately at -70°C. The vials were then lyophilized in a Usifroid (Paris) apparatus.

For use, each vial containing RBC was reconstituted with 0.5 ml of sterile distilled water, and 2.5 ml of sterile PBS containing 0.2% bovine serum albumin was added to obtain a final 1% RBC solution.

IHA test. Sera to be tested were first adsorbed with fresh sheep RBC overnight at 4°C. The next day, the RBC were removed by centrifugation at $1,000 \times g$ for 20 min.

IHA tests were performed in microplates as previously described (1). Sera to be tested were serially diluted 1:10 to 1:5120 in sterile PBS containing 0.2% bovine albumin.

In all IHA reactions, the following controls were included: a negative serum and two positive sera, one

of the high titer (1:1,280 to 1:2,560) and one of medium titer (1:320 to 1:640). The same positive and negative control sera were used throughout this study.

In parallel with CMV-RBC, each serum was also tested with nonsensitized RBC. In some comparative IHA tests, unfixed but washed, tanned CMV-RBC were used without being lyophilized.

CF. Complement fixation (CF) was done by using the microtiter system and commercial glycine-extracted CMV, strain AD 169-infected cell antigen.

EIA. Enzyme immunoassay (EIA) was performed by the technique already published for the measurement of herpes antibodies (2) with some modification (Denoyel et al., manuscript in preparation).

RESULTS

Critical steps in processing freeze-dried CMV-RBC. It is first important to screen sheep to find a suitable donor. To avoid nonspecific agglutination of fixed, lyophilized RBC, it was essential to treat the sheep blood mixed with Alsever solution directly with glutaraldehyde or Formalin. When the RBC were first separated from the plasma by centrifugation, washed, fixed, and freeze-dried, nonspecific agglutination in the IHA test was regularly observed. Even gentle centrifugation of the blood followed by resuspension of the RBC in the same supernatant plasma rendered the RBC unsuitable for subsequent fixation and lyophilization. These experiments were repeated several times, and the results consistently showed that it was critical to fix RBC in the whole blood plus anticoagulant solution. Such fixed RBC could then be tanned, sensitized with a viral antigen, freeze-dried, and used in IHA without agglutinating nonspecifically.

When the fixation was performed with glutaraldehyde, we found that the chemical purity of the reagent was a critical factor in obtaining specific agglutination. Only a pure concentrated product kept in sealed vials at 4°C gave satisfactory results in our hands. Even with such a pure product, it was necessary to check every batch of glutaraldehyde to avoid one producing nonspecific agglutination of fixed RBC.

The fixation of RBC by Formalin under the conditions described in Materials and Methods was easily and constantly accomplished by using a common, commercial product.

Another critical step in preparing fixed RBC for sensitization with a viral antigen was their treatment with tannic acid. Our results, in this respect, were in agreement with the findings of Yeager (6) concerning the choice of tannic acid concentration. Thus, optimal concentrations of tannic acid were found to be between 1:40,000 and 1:160,000, depending on the RBC donor sheep and the batch of viral antigen.

Freeze-drying of fixed RBC was carried out in PBS (pH 7) containing 0.2% bovine serum albumin and 5% lactose. The reconstitution of the dry product in distilled water was greatly facilitated by the presence of lactose.

Comparison of IHA performed with freeze-dried, CMV-RBC with CF and EIA. Two different groups of human sera were tested in two independent laboratories both by IHA performed with lyophilized, CMV-RBC and by CF. A good correlation in CMV antibody detection was found between these two techniques in both laboratories, as revealed by the coefficients of correlation ($r = 0.8041$ and $r = 0.8048$).

We also compared CMV antibody detection by IHA, CF, and EIA in a group of 31 randomly chosen human sera. All three methods detected the presence of CMV antibodies equally well. However, a difference was seen in the titers obtained by these methods. By using geometric mean titer ratios, EIA was found to be about 2.4 times more reactive than IHA, which was about 4.9-fold more reactive than CF.

Reactivity of freeze-dried CMV-RBC in IHA. The use of freeze-dried, virus-sensitized RBC in IHA raised the question of the reactivity of such a reagent in detecting CMV antibodies. To answer this question, we performed comparative IHA tests with (i) washed, tanned CMV-RBC; (ii) glutaraldehyde-fixed, tanned, CMV-sensitized, and lyophilized RBC; and (iii) formalin-fixed, tanned, CMV-sensitized, and lyophilized RBC.

In a series of 10 comparative IHA tests, we compared the antibody titers of two positive reference sera and negative control serum, by using simultaneously i, ii, iii, and control non-sensitized erythrocytes. The results are presented in Table 1. It can be seen that the geometric mean titers of IHA antibodies obtained with ii and iii were comparable to those obtained with i. However, a small statistically significant difference was noticed between iii and i, the former proving somewhat less reactive in these tests.

These results were confirmed in another experiment in which the three types of CMV-RBC were tested simultaneously against 58 selected human sera that were positive in CF (Table 2).

We found a very good correlation when we compared the geometric mean titer obtained with i and iii ($r = 0.9503$), and with i and ii ($r = 0.9431$). From the same experiment (Table 2), we can conclude that there was not a significant difference between the geometric mean titers obtained by using different types of CMV-RBC. The CMV antibody titers detected in IHA with freeze-dried, sensitized RBC were not different

TABLE 1. Comparison of two sensitization methods on the RBC behavior in IHA reaction with reference antibodies

Reference antibodies	No. of tests	CMV-RBC		
		Fresh	Lyophilized after fixation with:	
			Glutaraldehyde	Formalin
Positive serum 1	10	9.30 ^a (0.00)	9.00 (0.48)	8.30 ^b (0.82)
Positive serum 2	10	11.10 (0.43)	10.30 ^b (0.00)	10.10 ^b (0.63)
Negative serum	10	0	0	0

^a Geometric mean hemagglutination titers ($\log_2$) and standard deviations (in parentheses) for 10 tests with each category of RBC (fresh or freeze-dried after fixation with formalin or glutaraldehyde) against each reference serum.

^b Statistically significant different (t test, $P < 0.01$) geometric mean titer as compared to that obtained with fresh RBC taken as standard.

from those obtained with fresh CMV-RBC.

Thermostability of lyophilized CMV-RBC. Vials of lyophilized CMV-RBC from five different batches of ii and one batch of iii RBC were held in an incubator at 37°C. After various times of storage, vials were withdrawn, reconstituted, and run in an IHA test with one negative and two positive reference serum (Table 3). A control of freeze-dried, but nonsensitized RBC was also included in each experiment. All six batches of freeze-dried CMV-RBC were stable up to 9 months of maintenance at 37°C. Non-specific agglutination in the presence of control negative sera or of nonsensitized RBC was not observed.

Repeated IHA assays performed at various intervals with the same batch of freeze-dried CMV-RBC kept at 37°C showed little variation in titer (1 to 2 dilutions), which was not more than the inherent variation of the system.

DISCUSSION

IHA has multiple advantages as a routine procedure in the diagnosis of CMV (4) and in the screening of blood donors to decrease transfusion- and transplantation-associated CMV infections (6). Moreover, the possibility of using small blood samples collected on PKU (phenol-keto-urea) cards (1) and a microprocedure (3) make IHA the most suitable method for epidemiological studies.

Because long-term preservation of CMV-RBC is the main limitation of IHA, we developed a

TABLE 2. Capacity of CMV-sensitized, freeze-dried RBC to detect the presence of CMV antibodies in different human sera^a

CMV-RBC	GMT ^b (log ₂)	Standard deviation	<i>t</i> (level of significance)	Correlation coefficient (<i>r</i>) for individual samples
Formalin-fixed	8.97	2.54	0.3809 (<i>P</i> < 0.70)	0.9503
Fresh	9.16	2.82		
Glutaraldehyde-fixed	8.73	2.70	0.8416 (<i>P</i> < 0.40)	0.9431

^a IHA tests were performed with each kind of CMV-RBC with 58 human sera with different CMV antibody titers as determined by CF. The level of significance (*P*) of difference between two geometric means was evaluated by Student's *t* test.

^b GMT, Geometric mean titer.

TABLE 3. Stability of CMV-sensitized, freeze-dried RBC during storage at 37°C

RBC ^a batch	Reference sera ^b	Antibody titers ^c at month:						
		1	2	3	5	6	8	9
A	Pos. 1	640	640	320	640	640		
	Pos. 2	1,280	2,560	640	2,560	2,560		
	Neg.	0	0	0	0	0		
B	Pos. 1	640	320	320	320			640
	Pos. 2	1,280	640	1,280	1,280			1,280
	Neg.	0	0	0	0			0
J	Pos. 1	640		640			640	
	Pos. 2	2,560		1,280			2,560	
	Neg.	0		0			0	
K	Pos. 1	640	320	320	640			640
	Pos. 2	1,280	640	1,280	1,280			2,560
	Neg.	0	0	0	0			0
L	Pos. 1	640		320	640			
	Pos. 2	640		1,280	1,280			
	Neg.	0		0	0			
Y	Pos. 1	320			160		320	
	Pos. 2	1,280			640		640	
	Neg.	0			0		0	

^a The RBC were sensitized with CMV antigen after fixation with glutaraldehyde, except for batch Y, which was fixed with Formalin.

^b The titers of reference sera when tested in parallel IHA test with CMV-sensitized, fresh RBC were: positive serum 1 (Pos. 1), 640; positive serum 2 (Pos. 2), 1,280 to 2,560; and negative serum (Neg.), 0 (<20).

^c Reciprocal of the endpoint dilution as determined with sensitized, lyophilized RBC held at 37°C.

means of freeze-drying such RBC. The main obstacle in the preparation of stable reagents for IHA was nonspecific hemagglutination resulting from the RBC preparation procedure. It became clear that the cause of this nonspecific hemagglutination was fixation of RBC by glutaraldehyde or formalin after RBC had been separated from plasma by centrifugation. When whole sheep blood collected in AS was treated directly with glutaraldehyde or formalin, RBC could then be tanned, virus sensitized, and freeze-dried without showing subsequent nonspecific hemag-

glutination.

We have confirmed our previous results (1) and Yeager's findings (6) concerning the need for determining optimal tannic acid concentrations, which vary according to both the donor sheep and the batch of CMV antigen.

The use of Formalin for RBC fixation was less critical than glutaraldehyde treatment. However, the Formalin-fixed CMV-sensitized, freeze-dried RBC in the IHA test were somewhat less reactive than the glutaraldehyde-fixed reagent in detecting CMV antibodies.

It is important to stress that freeze-dried CMV-RBC were stable up to 9 months at 37°C. During this time their behavior in the IHA test did not change.

We found a remarkable correlation between IHA, CF, and EIA, illustrating the equal reliability of all three techniques in the detection of CMV antibodies. However, the levels of antibody detected were clearly higher in EIA and IHA.

Another advantage of IHA is for detection of those classes of antibodies which seems to appear first in CMV infection; IHA reactions were shown to correlate well with the presence of antibodies to CMV early antigens detected by immunofluorescence (5).

Yeager (6) simplified the IHA test for CMV by using human group O RBC fixed in glutaraldehyde and stored frozen at -70°C. With our method it was not possible to use human RBC since the method of fixation that we developed necessitated glutaraldehyde treatment of RBC directly in the whole blood. We could therefore not exclude the possibility that CMV antibodies present in human sera would bind to RBC during fixation. On the other hand, we did not find that glutaraldehyde-fixed cells reacted with the plastic of microtiter plates, enhancing nonspecific hemagglutination, as reported by Yeager (6).

At the present time, two laboratories collaborating in this study routinely use the procedure described above. They have found that IHA

performed with freeze-dried CMV-RBC is remarkable for its simplicity and high reproducibility.

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