A Simple Method of Preparing Protein-Erythrocyte Conjugates for Hemagglutination Tests.* (31739)

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The conjugation of proteins to erythrocyte surfaces has proved to be a valuable procedure for detection of antigens and antibodies(1-7). For this purpose, two well known techniques are often employed. The first consists of the treatment of erythrocytes with dilute tannic acid followed by addition of protein(1). This method is sensitive but tedious, since it requires daily preparation of new reagents. The second procedure involves initial contact between red cells and formalin with subsequent tanning and attachment of protein(4). Preparations of this sort are more stable, but, in our hands, lack sensitivity and reproducibility. Therefore, a simplified hemagglutination procedure was designed which involves tanning of the erythrocytes followed by coupling with protein in the presence of formalin. The cells coated in this manner have proved to be sensitive and stable for a period of 2 to 3 weeks. Initially a number of variables were studied such as pH, buffer systems, several concentrations of tannic acid, formalin and protein. The method to be presented incorporates the optimal conditions found for preparing protein-erythrocyte conjugates.

Methods. For hemagglutination with immune rabbit sera, the following steps were employed: Sheep or guinea pig erythrocytes were washed 3 times with 4-5 volumes of 0.85% saline and brought to a final concentration of 20%. An equal volume of 0.01% of an analytical grade of tannic acid in saline was added with thorough mixing. The cells were incubated at 37°C until frank agglutination had occurred, usually 10-30 minutes.† The erythrocytes were washed 3 times with 4-5 volumes of 0.15 M phosphate buffered saline, pH 7.2, following which the clumping disappeared. A final 20% cell concentration was prepared by addition of the same buffer.

An equal volume of the protein to be attached to the tanned cell was then added as a 1% solution in buffer. A similar volume of 1% neutral formalin in buffer was also added. The mixture was shaken, incubated at room temperature for 1 hour, and placed at 4°C overnight. On the next day the cells were washed 3 times with 4-5 volumes of buffer and an aliquot was brought to a concentration of 2% for use in the hemagglutination test. The remainder of the cells was placed in an equal volume of Alsever's solution and kept at 4°C. Samples were removed at weekly intervals and washed prior to testing. After the first week of storage, a gradually increasing degree of hemolysis was observed. Such cells, however, when washed with buffered saline, were fully sensitive in the hemagglutination test.

The following variations in the tanning of cells and coupling with protein were utilized for hemagglutination with immune human sera: For detection of rheumatoid factor, tanned sheep erythrocytes were mixed with equal volumes of 0.5% heat-aggregated (66°C, 30 min) Cohn FII globulin‡ and 1% neutral formalin in the usual manner. Following overnight storage at 4°C, cells were washed 3 times with 15 volumes of 0.15 M phosphate-buffered saline, diluted to a 2% suspension in buffer and stored at 4°C in this concentration. After one week in the refrigerator, an increasing amount of hemolysis was observed; nevertheless, these erythrocyte suspensions retained full sensitivity.

Hemagglutination tests with rabbit immune sera were carried out as follows: An initial 1:100 dilution was prepared in buffer; aliquots were kept frozen. Stock samples were thawed and titered at weekly intervals using 0.5 ml of 2-fold dilutions of the serum in buffer. Each tube then received 0.1 ml of a 2% suspension

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† For tanning human erythrocytes 0.1% was used for one hour at 37°C.

‡ Nutritional Biochemicals Corp., Cleveland, Ohio.

of washed, sensitized erythrocytes. The tubes were shaken, allowed to incubate for 1 hour at room temperature and then spun lightly in an angle centrifuge for 1 minute (500-1000 rpm). The last tube which showed distinct macroscopic agglutination when the cells were gently resuspended was taken as the endpoint of the titer. Since tanned and formalinized erythrocytes tend to stick together, centrifugation at higher speeds may result in false positive reactions. For this reason, appropriate control tubes were always included.

Each rheumatoid arthritis serum was heated 30 minutes at 56°C, and then absorbed twice with normal packed sheep cells to remove heterophile agglutinins. Thereafter 2-fold serial dilutions in a volume of 0.5 ml of buffer were prepared beginning at a 1:20 dilution. To each serum dilution, 0.1 ml of the 2% coated cells was added, the mixtures were shaken, and the tubes incubated at room temperature for 2 hours. Settling patterns were recorded as ++++, +++, ++, +, ± corresponding to those described by Heller and coworkers(8). The last tube showing a ± reaction was taken as the endpoint of the titer. When compared with those obtained following centrifugation, as described above, the settling pattern titers averaged 3.7 logs (base 2) higher.

Results. Six lots of freshly tanned, washed 20% suspensions of sheep erythrocytes were coupled separately with equal volumes of 1% ovalbumin (OA), 1% bovine serum albumin (BSA), 1% human serum albumin (HSA), 1% human gamma globulin (HGG), 1% keyhole limpet hemocyanin (KLH), and with 1% octopus hemocyanin (OH), in the presence of 1% formaldehyde in buffer as described. All samples were held in Alsever's solution at refrigerator temperature for the duration of the experiment. Aliquots were removed at weekly intervals, washed with buffered saline and made up to a concentration of 2% cells. High titered rabbit immune sera were diluted 1:100 and kept at —20°C. Samples were thawed and titrated, as described, in volumes of 0.5 ml, the initial serum concentration being 1:200. The results of hemagglutination tests done weekly for 4 weeks and finally after 60 days with the same lot of sensitized cells and immune serum are shown in Table I. It can be seen that with a variety of antigens and immune rabbit sera, the titers were reproducible for three weeks, but began to fall off significantly thereafter. Control tests for specificity consisted of titers of each immune serum against each heterologous antigen at an initial dilution of 1:400. Anti-KLH serum reacted weakly with OH-sensitized cells but not the reverse. Similarly, anti-HSA serum agglutinated the BSA antigen weakly at 1:400, but not at 1:800. Because of the sensitivity and specificity of the direct hemagglutination reactions with high titer rabbit immune sera, we did not attempt to use settling patterns though good buttons were obtained in control tubes of cells sensitized with all antigens except OH. As expected, the settling titers exceeded the direct hemagglutination values by 3 to 5 logs (base 2). Since tube 10 represented a dilution of 1:102,400, it was calculated from quantitative precipitation data, that we were detecting approximately 0.04 μg of antibody protein per ml of serum.§

TABLE I. Agglutination of Tannic Acid Formalinized Sheep Erythrocytes Conjugated with a Variety of Antigens by Immune Rabbit Serums. Stability of erythrocyte conjugates.

Antigens	Age of coated cells (days)					
	1	7	14	21	30	60
BSA	9*	10	10	8	8	5
HSA	11	10	11	10	9	8
OA	11	9	11	11	10	10
HGG	6	4	6	5	5	5
KLH	12	11	12	9	10	7
OH	15	16	17	16	nd†	nd

* Titer (log base 2).

† Not done because cells agglutinated spontaneously after 30 days storage.

The technique of determining titer by examination of the settling pattern was applied to an investigation of rheumatoid factor levels in 10 sera from patients with classical rheumatoid arthritis(9), and in 5 sera from normal subjects. Results are shown in Table II. The tannic acid, formalin-treated sheep erythrocytes (TAF test) were titered 1, 3, 9, and 17 days after attachment of the denatured Cohn FII globulin. The data are compared with those obtaind using a standard tanned

§ We are indebted to Drs. Arthur Malley and Alfred Amkraut, Oregon Regional Research Primate Center, for KLH and OH reagents together with the quantitative serum assays.

TABLE II. Agglutination of Tannic Acid Formalinized (TAF) Erythrocytes Conjugated with Human FII by Rheumatoid Arthritis (RA) Sera. Stability of erythrocyte conjugates and relationship of titers to tanned cell hemagglutination(8) and sensitized sheep cell agglutination (SSCA) (10) methods.

RA serums	TAF-FII hemagglutination titer* Age of coated cells (days) 1	3	9	17	FII tanned* cell tube titer‡	SSCA† titer
E. McB.	9	8	10	8	10	5
A. R.	8	7	7	7	8	4
H. C.	10	10	11	10	11	4
D. Z.	9	9	11	9	10	5
M. R.	7	7	6	6	7	3
S. C.	9	9	9	9	8	5
N. B.	7	7	7	6	7	4
M. G.	10	10	10	10	11	8
A. K.	12	12	11	12	12	9
J. G.	12	11	11	11	11	7
5 normal sera	0	0	0	0	0	<3

* First tube = 1:20 dilution.
† First tube = 1:7 dilution.
‡ Five mg denatured FII per ml 33% tanned cells. Phosphate-buffered saline, pH 8.0, used throughout.

cell hemagglutination technique(8), and with the less reactive sensitized sheep cell agglutination (SSCA) procedure(10). Comparison between TAF and the tanned, nonformalinized cell titrations revealed good correspondence. Normal sera, on the other hand, remained nonreactive. Of special interest is the fact that titration values with the same sera on 4 occasions within a period of 17 days did not vary significantly, thus attesting to the stability of the TAF-FII antigen. From this and other experiments, we have concluded that the TAF method of coating erythrocytes is a suitable substitute for the standard tanned cell agglutination procedure and has the distinct advantage of increased stability.

Discussion. The decision to use both tannic acid and formaldehyde for purposes of fixing protein to the surface of erythrocytes was based on observations in the literature(3,4). Thus Fulthorpe(3) attached tetanus toxoid to tanned sheep erythrocytes and stabilized the complex in a serum-buffered saline containing 20% formaldehyde. Ingraham(4) reported that BGG coupled directly with washed human erythrocytes in the presence of 10% formalin-saline. The resultant product was stable, suitable for both hemagglutination and photometric quantitation(7). Our hemagglutination procedure was originally designed to provide stable reagents for a period of 1 week. The formaldehyde was added in low concentration and for a brief period in order to insure maximal sensitivity and to fix the protein to the erythrocyte surface. It was also known that tannic acid treated erythrocytes are fragile and it was, therefore, hoped that the brief contact with formalin would increase their stability.

At present it is not known whether the protein binding is irreversible. The fact that the sensitized erythrocytes maintain constant titers for over 2 weeks suggests this may well be so. The stability of the sensitized erythrocyte suspension is a desirable characteristic and allows for preparation of batches of antigen which will be usable for predictable periods of time so that each new preparation can be easily standardized against the old. The sensitivity of the reaction observed when mixtures were centrifuged and resuspended was approximately 0.04 μg of antibody protein per ml of serum. This order of activity could be increased to the reported level of 0.001-0.002 μg(7) by utilizing the settling patterns for determining end points. In the case of the rheumatoid factors, the hemagglutination can be attributed to the 19 S component(11) whereas the titer with rabbit anti-KLH serum reflects the predominant 7 S type of antibody usually associated with late responses to this antigen. In fact, hemagglutination is known to occur with both 7 S and 19 S immunoglobulins(7).

With fully formalinized and tanned cells, Daniel and coworkers(6) observed that optimal coupling was pH dependent and usually occurred between 4.6 and 7.2 with a characteristic range for each antigen. The finding that adequate attachment of a variety of proteins to erythrocytes occurred at the single pH of 7.2 came as a pleasant surprise. In initial tests, we had successfully coupled BSA at pH 6.5. Similarly, with denatured FII globulin, good coupling was obtained at pH 8. Both proteins attached equally well at pH 7.2. These features of the reactants allowed the use of a single buffer and a uniform coupling

technique which simplified procedures considerably.

Each of the sera from the patients with rheumatoid arthritis was inactivated at 56°C for 30 minutes and absorbed twice with packed sheep erythrocytes to remove low titered heterophile agglutinins. With the immune rabbit sera, specificity was checked beginning at a dilution of 1:200 without preliminary absorption. We were not concerned with heterophile agglutinins because all sera were from highly immunized animals and could, therefore, be tested beyond the normal range for this antibody. We did, however, check 2 sera, an anti-M and an anti-ovalbumin beginning at a titer of 1:4 and continuing to 1:1028. Neither direct hemagglutination nor abnormal settling patterns occurred. With normal mouse and guinea pig serum, however, titers as high as 1:128 were obtained against tannic acid treated and formalinized sheep erythrocytes alone and also with similarly prepared cells to which 1% BSA had been coupled. With these and with some selected rabbit antisera, preliminary absorption with sheep erythrocytes may be necessary.

Summary. A simple method has been described which allows firm coupling of a variety of proteins to tanned sheep erythrocytes in the presence of formalin. Cells, so treated, agglutinate strongly and specifically with antibody; they also form suitable settling patterns and maintain specific reactivity and sensitivity for a full 2 weeks. Agglutination with immune rabbit sera with as little as 0.04 μg of antibody protein per ml serum was readily detected. The tannic acid-formaldehyde (TAF) test compared favorably with 2 other established hemagglutination procedures for detecting rheumatoid factor in 10 sera from patients with rheumatoid arthritis.

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Suppression of Interferon and Antibody and Multiplication of Newcastle Disease Virus in Cytomegalovirus Infected Mice.* (31740)

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Studies of mice infected with mouse cytomegalovirus (MCMV) showed that during the first 2 weeks of infection, animals were markedly inhibited in their capacity to produce circulating interferon in response to intravenous injection of Newcastle disease virus (NDV)(1). This finding suggested that NDV might be handled differently by MCMV-infected mice.

NDV penetrates mouse cells(2) and is a potent stimulator of mouse interferon(3,4). However, it does not normally multiply in mouse tissue. Ginsberg(5) showed that large quantities of NDV administered intranasally produced a toxic pulmonary consolidation in Swiss white mice. Inactivated virus did not induce the pathology, and although infectious virus was necessary, it was rapidly cleared

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