Effect of Tannic Acid and Adsorbed Purified Blood Group Substances on Human Erythrocytes.* (24170)

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During recent years, many immunological tests have been described in which use was made of tannic acid-treated erythrocytes to adsorb various antigens from solution. It was our purpose to study the effect of tannic acid on the red cell itself by observing changes in serological reactivity of the A, B, C, D, E, M and N antigens of human erythrocytes following the tanning process. In addition, tanned human red cells were studied for their ability to adsorb purified blood group A and B substances; this portion of the work is an extension of the findings of Brading(1) who found that blood group A and B substances could be adsorbed onto group O cells.

Materials. Several diluents were employed: 0.85% NaCl unbuffered or mixed with equal volume of $\frac{M}{15}$ $Na_2HPO_4 \cdot KH_2PO_4$ solutions to produce pH 7.2 or 6.4; and serum-saline prepared by mixing with 0.85% saline serum from a person having type AB blood to final serum concentration of 0.4 or 1%. The human A substance (cyst 9 phenol-soluble fraction) was isolated from a human ovarian cyst fluid(2) by phenol extraction method of Annison and Morgan(3); the human B substance, also of cyst origin, was obtained through the kindness of Dr. Sidney Leskowitz of Mass. General Hospital. Each week a 1:1000 stock solution of Merck USP fluffy tannic acid was prepared and daily working solutions were made by dilution of this stock, using 0.85% NaCl. Human red cells of types O, A, and B were collected directly into acid citrate dextrose preservative (ACD) solution, stored at 0°-4°C and used until they were 3 weeks old, since it was determined that cells over 4 weeks old were too fragile for use. Cells to be tested for factors C, D, E, M, and N were used within 2 hours after collection. All antisera were commercially prepared, "saline agglutinating" typing sera and all were of human origin except anti-M and anti-N which were rabbit antisera.

Method. Red cells were washed 3 times in saline and a 2.5% suspension prepared in buffered (pH 7.2) saline. An equal quantity of appropriately diluted tannic acid solution was added to this suspension, which was then shaken and incubated in water bath at 37°C for 15 minutes. The cells were packed by centrifugation for 2 minutes at about 1300 rpm and washed once in buffered (pH 7.2) saline. A solution of the blood group substance in buffered saline (pH 6.4) was added to the packed tanned cells in volume sufficient to give a 4% cell suspension. After 15 minutes at 37°C for adsorption the cells were washed once in 0.4% human AB serum as recommended by Brading(1) to prevent hemolysis and aid in resuspension. For agglutination, a 2.5% cell suspension was made in 0.4% AB serum and to 0.2 ml of this was added 0.2 ml of antiserum diluted in 1% AB serum; where required, controls of untreated cells and cells treated with tannic acid alone were used. The tubes were shaken, incubated 30 minutes at 37°C, reshaken and centrifuged for 2 minutes at about 1300 rpm, then examined macroscopically and at magnification of 30x. The results were recorded as follows: 4+, a single heavy clump of packed cells that did not disperse on gentle shaking; 3+, several gross clumps; 2+, clumps small but clearly visible macroscopically; 1+, clumps well defined at 30x, all cells clumped; ±, scattered clumps at 30x; —, no clumps at 30x, cells evenly dispersed on gentle shaking. In titrating with anti-D serum, the saline tube procedure was followed as outlined by American Asso. of Blood Banks(4); the results were recorded as for anti-A and anti-B titrations. In the case of C and E antigens, 2 drops of a 2.5%

* Supported by grant of Nat. Inst. of Allergy and Immunol. and by Schwartz Fund, Tulane Univ. Medical School.

cell suspension were added to 1 drop of antiserum, shaken, incubated at 37°C for one hour and centrifuged 1 min. at about 1000 rpm; the results were reported as positive or negative after examination with 10x hand lens. M and N antigens were tested in similar mixtures after incubating 30 minutes at room temperature, and examined immediately for clumps without centrifugation; the results were recorded as positive or negative.

Results. To test the effect of tanning on surface antigens, human red cells possessing antigens A, B, C, D, E, M, and N were treated with a 1:20,000 dilution of tannic acid. The cells were then tested for ability to agglutinate with homologous antiserum. When tested against anti-A, anti-B, and anti-D, the tanned cells agglutinated as well as untanned cells and the titers of antisera were the same in each case. In contrast, the anti-E, anti-M, and anti-N sera failed to agglutinate tanned cells; since these commercial antisera were relatively weak, it was not practical to carry out a titration and the possibility, therefore, cannot be ruled out that a sufficiently potent antiserum might have given some agglutination. Brading(1) found that tanning the cells reduced anti-A, anti-B and anti-D titers; we have no explanation for the difference between her results and ours.

Before extensively studying adsorption of purified blood group substances onto tanned cells, a single system (A substance adsorbed onto O cells) was studied to determine the most suitable concentration of tannic acid and blood group substance. Cells treated with tannic acid in concentrations varying from 1:2000 to 1:40,000 and then incubated with 1% solution of A substance all gave the same (1:32) titer with anti-A serum. However, when the A substance was added, there was some hemolysis in those cells treated with tannic acid concentrations of 1:10,000 or higher. Since the use of tannic acid concentrations greater than 1:20,000 did not increase the reactivity of the cells toward anti-A serum, this concentration was selected for sensitization in subsequent work.

Group O cells treated with tannic acid 1:20,000 were then incubated with blood group A substance in concentrations varying from 0.01% to 2%. It was found that the anti-A titer was constant when the blood group concentration used for adsorption varied from 0.1% to 2%, but was distinctly reduced at the 0.01% level. It should be noted that untanned O cells did not adsorb blood group A substance. From this experiment a concentration of 1% blood group substance solutions was considered to be optimal.

TABLE I. Effect of Adsorption of Blood Group Substances on Erythrocytes of Groups A and B.

Cells	Titer *vs* anti A	Titer *vs* anti B
Untreated A cells	1/64	
A cells + 1% B sub.	"	
Tanned A cells	"	
Idem + 1% B sub.	1/16	1/32
Untreated B cells		1/256
B cells + 1% A sub.		"
Tanned B cells		"
Idem + 1% A sub.	1/16	1/32

Note: Titer represents highest dilutions giving a 1+ reaction.

Tanned group A cells were then treated with B substance, tanned group B cells with A substance, and both groups of cells were then tested against anti-A and anti-B sera; the results are recorded in Table I. The adsorption of blood group substance onto tanned cells resulted in a lowered antiserum titer against the antigen originally contained on the red cell and the added ability to agglutinate with the antiserum to the adsorbed antigen. There was no change in serum titer to the original or adsorbed antigens when concentration of blood group substance was increased from 1 to 2%. The group A and group B cells, therefore, simulated group AB cells in their reactivity.

Since in both cases studied there was a partial suppression of the reactivity of the original erythrocyte antigen, the question was raised whether or not this was specific or some general effect on reactivity of the red cell. B substance was adsorbed onto A cells possessing the D antigen, and from Table II it can be seen that reactivity of the D antigen was unaffected.

Discussion. Since many antigens are known to be present on, or close to, the surface of the red cell, they must obviously oc-

TABLE II. Effect on Anti-D Titer of Adsorption of Blood Group B Substance.

A cells	Titer of anti-D serum						
	U	1/2	1/4	1/8	1/16	1/32	1/64
(D) + cells in saline	3	4	4	3	2	2	—
(D) + cells in tannic acid	3	4	3	3	2	2	—
(D) + cells, tanned, + 1% B	4	4	3	3	2	2	—
(D) + cells, + 1% B	4	4	4	3	2	1	—
(d) − cells in saline	—	—	—	—	—	—	—
(d) − cells in tannic acid	—	—	—	—	—	—	—
(d) − cells, tanned + 1% B	—	—	—	—	—	—	—
(d) − cells + 1% B	—	—	—	—	—	—	—

cupy limited and specific sites. Little is known concerning distribution of these antigens or whether they occupy one or more areas, although it may be presumed that if a cell can agglutinate that the antigen involved occupies at least 2 distinct sites. Tanning of the red cells sorted out the antigens tested into those that were unaffected by such procedure (A, B, and D antigens) and those whose activity as determined by reaction with homologous antisera was eliminated or markedly reduced (C, E, M, and N antigens). This would seem to indicate that the tannic acid was attached on or close to the latter 4 antigens; it further points up a difference in location of the 3 Rh antigens tested, the C and E antigens being quite distinct from the D antigen.

The A, B, and D antigens, while unaffected by tannic acid, react differently when the A and B substances are adsorbed onto the tanned cells. The A and B antigens already part of the cell surface are somewhat reduced in activity while the D antigen is unaffected. One possible explanation is that the adsorbed substances partially block the indigenous antigen but that the D antigen is sufficiently remote so that its activity is undisturbed.

It is interesting to note that adsorption of blood group substances onto red cells does not take place unless the cells are tanned. Since in most instances polysaccharides will adsorb to untanned cells, it would appear that the polypeptide portion of the blood group substance is probably important for the adsorption process while apparently unnecessary for the serological specificity. It is of further interest that adsorption of A or B substances onto cells of group B or A converts them to cells indistinguishable serologically from cells of group AB.

Summary. When human erythrocytes were treated with tannic acid, they reacted normally with anti-A, and anti-D sera, but the reactions with anti-C, anti-E, anti-M and anti-N were either eliminated or markedly reduced. Purified blood group A substance could be adsorbed onto tanned B cells and B substance onto tanned group A cells; in each case the cells reacted serologically as group AB cells. When the D antigen was present along with the A antigen, the former was unaffected by adsorption of B substance.

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Received June 2, 1958. P.S.E.B.M., 1958, v98.