Effect of Glutaraldehyde Fixation on Erythrocyte Agglutinability[1] (37732)

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Glutaraldehyde (GA) is widely used as a fixative in electron microscopy (1, 2). Erythrocytes have been employed as models to study alterations occurring during fixation. Morel *et al.* (3) have indicated that the red cell membrane is highly permeable to GA and that the selective permeability to ions is lost by fixation. Herz and Kaplan (4) have shown that GA causes membrane modifications associated with the irreversible inactivation of acetylcholinesterase, an enzyme located at or near the cell surface (5, 6). These findings lead to the suggestion (4) that GA may also affect other surface-related characteristics of the red cell such as electrophoretic mobility and agglutinability. Vassar *et al.* (7) have recently reported that GA increases the surface negative charge and that this change does not involve *N*-acetylneuraminic acid, which is responsible for the electrokinetic behavior of normal erythrocytes in an electrolyte medium (8). The purpose of this paper is to show that GA fixation interferes with the agglutination of red cells by homologous antibodies without significantly affecting antibody uptake.

Materials and Methods. Preparation of red cells and GA treatment. Blood obtained from normal adults was collected with EDTA, and the erythrocytes were washed three times with 20 vol of ice-cold 0.1 *M* sodium–potassium phosphate buffer, pH 8.0. The supernatant and buffy coat were removed by suction, and an approximately 50% (v/v) red cell suspension was prepared after the last centrifugation. A 50% (w/w) GA solution (biological grade, Fisher Scientific Co.) was diluted in 0.1 *M* sodium–potassium phosphate buffer, adjusted to pH 8.0, and used immediately. The concentrations of GA employed were 5, 0.5, 0.05, and 0.005 mg/ml. Twenty volumes of these GA solutions were added to 1 vol of cell suspension at 4° and incubated for 20 min at 25°. Cells treated with buffer alone were used as controls. Following incubation, erythrocytes were washed five times with 100 vol of buffer.

Agglutinability. Agglutinability of GA-treated cells was ascertained with human anti-A and/or anti-B serum (Ortho Diagnostics) and judged on a 1 to 4 scale. In some experiments, blood-group-A or blood-group-B erythrocytes were incubated at 22° with homologous human antiserum in amounts insufficient to cause direct agglutination, but sufficient to produce agglutination with rabbit anti-human globulin (Coombs) serum. Cells thus treated were washed five times with phosphate buffer and then incubated for 20 min with various concentrations of GA. Following this treatment and multiple washings with buffer, agglutinability by rabbit anti-human globulin serum was determined.

Antibody binding. For the study of antibody binding capacity, blood-group A or B erythrocytes were exposed to 5, 0.5, and 0.05 mg/ml of GA as indicated above. Cells treated with buffer alone and blood-group O erythrocytes served as controls. Following 5 washings with phosphate buffer, the cells were incubated for 30 min at 22° with anti-A or anti-B serum and subsequently sedimented by centrifugation. The supernatant was diluted and tested by agglutination for the presence of residual anti-A or anti-B anti-

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TABLE I. Effect of GA on Agglutinability of Group-A Erythrocytes and A Erythrocytes Coated with Anti-A Antiserum.

GA (mg/ml)	Agglutination of A cells by anti-A serum[a]	Agglutination of antibody-coated cells by anti-human globulin serum[b]
5	0	0
0.5	0	0
0.05	2+	2+
0.005	4+	4+
None	4+	4+

[a] Washed group-A red cells were exposed to GA as indicated in text, washed, and tested for agglutinability with anti-A serum.

[b] Washed group-A red cells were incubated for 30 min at 22° with 1:5000 dilutions of anti-A serum. No agglutination was noted. After 5 washings with 0.1 *M* phosphate buffer, pH 8.0, cells were exposed to GA for 20 min at 25°. Following 5 washings with buffer, agglutinability was determined with rabbit anti-human globulin (Coombs) serum.

bodies using a standard 5% suspension of A_1 or B erythrocytes (Affirmagen, Ortho Diagnostics). Other details are indicated in Table II.

Results. As seen in Table I, group-A erythrocytes treated with 0.5 mg/ml (5×10^{-3} *M*) of GA were not agglutinated by homologous antibody. However, although diminished in intensity, agglutination was noted with cells exposed to 0.05 mg/ml of the reagent. The action of GA was irreversible: no regeneration of agglutinability was noted after washing the cells for 20 times with phosphate buffer, 0.15 *M* NaCl, or 0.15 *M* NaCl solution containing 1% of bovine serum albumin. Identical results were obtained with group-B and group-AB erythrocytes. The GA-fixed cells retained normal morphological appearance and were not lysed by water, repeated freeze-thawing, and prolonged ultrasonication with a Raytheon 10-kc oscillator.

The markedly reduced agglutinability of GA-treated cells suggested alterations of the specific antigenic receptors. To test this possibility, the antibody binding capacity of erythrocytes fixed with various concentrations of the reagent was determined. As seen in Table II, despite their nonagglutinability, group-A erythrocytes, treated with 5 and 0.5 mg/ml of GA prior to their exposure to excess anti-A serum, removed almost as much antibody as cells treated with 0.05 mg/ml or as control cells incubated with phosphate buffer alone. The possibility of a nonspecific adsorption was eliminated by finding no removal of anti-B antibodies by GA-treated A erythrocytes and vice versa.

Since these findings indicated that GA did not destroy the receptors that determine ABO specificity, it was of interest to ascertain the effect of the reagent on the interaction of antibody-coated erythrocytes with rabbit anti-human globulin (Coombs) serum. Hence, blood-group-A erythrocytes were sensitized with dilute anti-A serum and then treated with various concentrations of GA at 25° and pH 8.0. As can be seen in Table I, sensitized cells treated with 5 and 0.5 mg/ml

TABLE II. Removal of Anti-A Antibodies by GA-Treated A Erythrocytes.[a]

Dilutions of supernatant	A erythrocytes treated with: Phosphate buffer	Glutaraldehyde (mg/ml) 5	0.5	0.05	Group-O red cells
1: 2	3+	4+	4+	4+	4+
4	2+	2+	2+	2+	4+
8	1+	2+	1+	1+	4+
16	0	1+	(±)	(±)	4+
32	0	0	0	0	4+
64	0	0	0	0	3+

[a] Washed group-A red cells were treated with various concentrations of GA and phosphate buffer for 20 min at 25° and pH 8.0. After 5 washings with buffer, 1.0 ml of human anti-A serum was added to 0.2 ml of GA-treated A erythrocytes, phosphate-treated cells (positive control), and blood-group-O erythrocytes (negative controls). No agglutination was noted with A cells exposed to 5 and 0.5 mg/ml of GA. After 30 min at 22°, cells were centrifuged at 800*g* for 5 min, the supernatant was diluted as indicated, and tested for residual anti-A antibodies using a standard 5% (v/v) suspension of A_1 erythrocytes. 0 indicates absence of residual anti-A antibodies.

of GA were not agglutinated by rabbit antihuman globulin serum, but cells exposed to 0.05 mg/ml were agglutinated, although to a lesser extent than controls. Similar results were obtained when group-B erythrocytes were sensitized with anti-B serum prior to treatment with GA. These findings indicate that this reagent not only interfered with agglutinability of red cells by homologous antisera but also hindered the agglutination of antibody-coated cells.

Discussion. The foregoing serological findings confirm and complement the recent experiments by Matsukura (9), who obtained qualitatively similar results by applying immunoelectron microscopic techniques to erythrocytes fixed with 25 mg/ml of GA for prolonged periods of time (2 hr–1 week). As with the inactivation of acetylcholinesterase (4), the effect of GA on red cell agglutination is irreversible; repeated washing of the fixed cells does not restore agglutinability. This observation can be contrasted with the reported regeneration of agglutinability by ABO- and MN-specific antibodies after extensive washing of formaldehyde-treated erythrocytes (10). The failure to restore agglutinability is in keeping with the action of GA on other systems, reflecting the reactivity of this reagent and its tendency to form stable derivatives.

Although it has been suggested that the bifunctional GA acts through α, β-unsaturated polymeric forms (11) in the formation of cross-linked derivatives, Vassar *et al.* (7), based on the positive Schiff reaction of GA-treated red cells, postulate that the increase in negative surface charge may indicate that GA acts at some sites of the peripheral zone of the erythrocyte as a unifunctional reagent. Such an interpretation may also apply to the observation that GA treatment prevents agglutination by hindering the formation of intercellular bridges which bind the red cells together in a lattice of agglutinated particles, essential for the second stage of agglutination. The finding that red cells fixed with GA can remove specific antisera from solution without being agglutinated is similar to the effect observed with formaldehyde (9, 12, 13) and with tannic acid (14), but differs from that seen with 1,5-difluoro-2,4-dinitrobenzene (15). The latter not only depresses agglutinability but also prevents antibody uptake by forming stable cross-linked dinitrophenylene derivatives with the red cell membrane.

Summary. The fixation of human erythrocytes with glutaraldehyde causes a striking reduction in the agglutinability by ABO-specific antisera. However, glutaraldehyde does not appear to destroy the specific antigenic receptors, since the fixed cells retain the capacity to adsorb specific antibodies from solution.

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