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Research Article

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On "Incomplete" Anti-Rh Antibodies: Mechanism of Direct Agglutination Induced by Mercaptoethanol *

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Since "incomplete" or "blocking" antibodies were first demonstrated in 1944 (1, 2), a variety of techniques has been developed to demonstrate the combination of incomplete antibodies with the appropriate erythrocytes (3). These procedures utilize high protein media, enzyme-treated cells, the antiglobulin (Coombs') reaction, hypotonic environments, or isotopic labeling.

Previous explanations for the absence of direct agglutination of erythrocytes by "incomplete" antibodies dealt with the antigenic nature of the erythrocyte surface and the valency of the antibody molecule. Most investigators have concluded that the incomplete antibody molecule is bivalent (4–7). A more recent approach concerns the orientation of the combining sites of the incomplete antibody molecule. Pirofsky and Cordova (8, 9) postulated that the incomplete anti-Rh antibody molecule has one reactive group available for lattice formation and one or more potentially reactive groups that are not available because of steric inaccessibility. Their experiments showed that in the presence of mercaptoethanol incomplete anti-Rh serum produced direct saline agglutination of the appropriate cells. This finding suggested that the reduction of disulfide bonds alters the tertiary structure of the antibody molecule in such a way as to expose the blocked sites, which are then available for agglutination of erythrocytes in a saline medium.

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Since there is little, if any, precedent to suggest that the incomplete anti-Rh antibody molecule differs structurally from other 7 S agglutinating or precipitating antibody molecules, we decided to investigate this problem further. Our data, although not in accord with the original postulate of Pirofsky and Cordova (8, 9), do suggest alternative explanations for the phenomenon they described.

Methods

Anti-Rh serum. The various anti-Rh sera used in this investigation were obtained from donors in the San Francisco Bay area. Other sera were kindly supplied by Drs. Nicholas Costea, Herbert Perkins, and Marian Waller.

Serological techniques. 1) Erythrocytes. Whole blood was obtained at weekly intervals from an O Rh-positive donor, mixed with an equal volume of Alsever's solution, and stored at 4° C. Before each experiment, the cells were washed three times in saline and resuspended to 2% of the packed cell volume. In one experiment the washed cells were adjusted to a 2% suspension and incubated at room temperature with an equal volume of either 1.0 M or 0.1 M 2-mercaptoethanol (ME) [1] for 15 and 60 minutes, respectively. The cells were then centrifuged, and the supernatant containing the reducing agent was decanted. The packed cells (0.2 ml) were immediately washed three times with 10-ml vol of saline and finally resuspended in saline at a concentration of 2%. The amount of reducing agent remaining after the third wash was calculated to be less than 10^{-5} moles per L.

2) *Agglutination.* Serial twofold dilutions of the serum or γ-globulin preparations [2] were added to equal volumes (0.1 ml) of 2% suspensions of the cells in 10- × 75-mm test tubes. After incubation for 1 hour at 22° C, the tubes were centrifuged for 1 minute at 1,000 rpm in a Clay-Adams centrifuge. Agglutination was scored as 0 to 4+ after gentle tapping of the packed cells.

When agglutination was scored in the presence of ME, sensitization was carried out at room temperature for 5 minutes. Appropriate controls were included in each experiment.

[1] Matheson, Coleman and Bell, Norwood, Ohio.

[2] A 1:2 dilution of the γ-globulin preparations contains 5 mg of protein per ml.

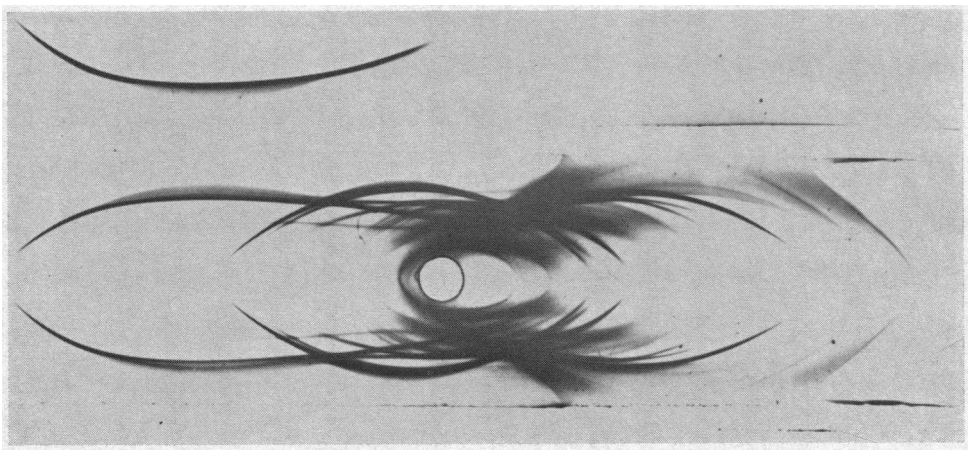

FIG. 1. IMMUNOELECTROPHORETIC PATTERNS OF RIP ANTI-RH γ-GLOBULIN. The upper well was filled with Rip anti-Rh γ-globulin (10 mg per ml) and the lower well with normal human serum. The bands were developed with rabbit antiserum to whole human serum.

Preparation of the 7 S γ-globulin. The γ-globulins from one anti-Rh serum and from one normal serum were prepared by the combined procedures of Kekwick (10) and Sober, Gutter, Wyckoff, and Peterson (11). After two successive precipitations with sodium sulfate, final concentrations of 18 and 14%, respectively, the precipitate was redissolved in saline and dialyzed overnight in the cold against 3.8 L of saline. The protein was then dialyzed against 3.8 L of 0.01 M sodium phosphate buffer, pH 8.0, clarified by centrifugation, and applied to a 100-ml column [3] of DEAE-cellulose [4] previously equilibrated at pH 8.0 in 0.01 M phosphate buffer. The γ-globulin was eluted with the same phosphate buffer, concentrated by pervaporation, and dialyzed against 3.8 L of saline. In all experiments, except where indicated in the text, the dilutions of the protein were made with physiological saline. The γ-globulin obtained in this manner exhibited a single peak in the ultracentrifuge and had a sedimentation coefficient ($s_{20,w}$) of 6.8.[5] No serum contaminants could be demonstrated by immunoelectrophoresis (Figure 1). In each case, 5.8 mg of γ-globulin was recovered per ml of the original serum.

[3] In preliminary experiments, a 100-ml column of DEAE-cellulose was sufficient to remove serum proteins other than γ-globulin from 1 g of protein obtained by the Kekwick procedure (10).

[4] Schleicher and Schuell, Keene, N. H.

[5] The activity of the purified 7 S anti-Rh preparation, which was at a concentration of 14 mg per ml, rapidly deteriorated after storage at 4° C for 1 week. Re-examination of the material by immunoelectrophoresis and ultracentrifugation showed considerable breakdown of the 7 S proteins to smaller fragments. These observations reinforce those of James, Henney, and Stanworth (12) and emphasize the care that must be exercised in testing purified 7 S γ-globulin preparations for immunological purity and activity.

Reduction and alkylation of 7 S anti-Rh γ-globulin. Thirty-three mg of the 7 S anti-Rh protein in 3.3 ml of saline was treated with ME at a final concentration of 0.1 mole per L for 1 hour at 37° C. The reduction was stopped by adding iodacetamide [6] to a final concentration of 0.1 mole per L. The reduced and alkylated protein was then dialyzed against 3.8 L of saline. This procedure resulted in approximately 30% aggregation of the 7 S protein (Figures 2 and 3). The reduced and alkylated protein (3.3 ml) was then applied to a 150-ml column of Sephadex G-200.[7] The protein was eluted with saline in 3.0-ml fractions.

Protein determinations. Protein concentrations were estimated from the optical density of the solutions in saline at 280 mμ. Extinction coefficients of 15 and 6.6 were used for 1% solutions of γ-globulin and ovalbumin, respectively.

Analytical techniques. Sedimentation velocities were measured in a 4° 12-mm cell in the Spinco model E ultracentrifuge at 59,780 rpm. Immunoelectrophoresis was performed according to the micromethod of Scheidegger (13) using barbital buffer, pH 8.6, $\mu = 0.075$.

Other materials. Bovine γ-globulin (BGG) was used as the dry Cohn Fraction II preparation and ovalbumin (EA) as the twice crystallized preparation.[8]

Results

Effect of 2-mercaptoethanol on the saline activity of anti-Rh sera. Eleven anti-Rh sera of varying titers and specificities were tested. Serial twofold dilutions of the sera were incubated with equal volumes of a 1.0 M solution of ME at room

[6] Mann Research Laboratories, New York, N. Y.

[7] Pharmacia, Uppsala, Sweden.

[8] Mann Research Laboratories, New York, N. Y.

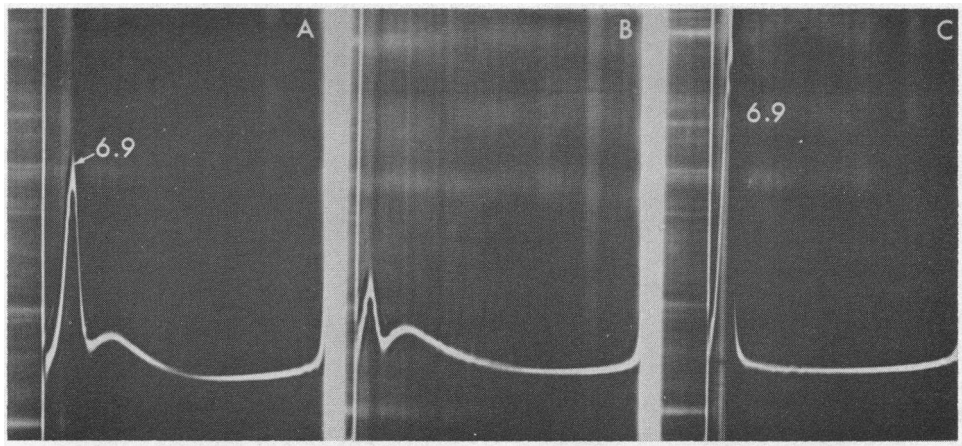

FIG. 2. SCHLIEREN ULTRACENTRIFUGAL PATTERNS OF REDUCED AND ALKYLATED ANTI-RH γ-GLOBULIN. Samples in sodium chloride, $\mu = 0.15$. Sedimentation is from left to right at 59,780 rpm. *A*) Rip anti-Rh γ-globulin reduced and alkylated at 16 minutes. *B*) Same as A, separated on Sephadex G-200, peak 1 at 0 time. *C*) Same as A, separated on Sephadex G-200, peak 2 at 0 time. The numerals are $s_{20,w}$ values. The s value of the slower component in B could not be accurately measured; however, the mobility corresponded closely to that of C.

temperature for 15 minutes. Here, as in subsequent experiments in which ME was used, agglutination was scored 5 minutes after the addition of the cells. In a separate series of dilutions, the saline and antiglobulin titers were determined concurrently; the cells were allowed to react with the anti-Rh sera for 1 hour at room temperature. The results, which confirm and extend the observations of Pirofsky and Cordova (8, 9), clearly show a correlation between the antiglobulin titer and the ability of ME to induce saline activity by the incomplete anti-Rh sera (Table I). Five of the eleven sera tested had significant saline activity in the presence of ME. Of the five, all had antiglobulin (Coombs') titers of 1:256 or greater, and two had saline activity in the absence of ME. One anti-Rh serum (Wash anti-D) had an antiglobulin titer of 1:256, but showed no significant saline activity in the presence of ME. The antiglobulin titers of the anti-Rh sera were not affected by treatment with ME. In each instance where ME induced demonstrable saline activity, washing the cells three times with 10-ml vol of saline resulted in a reduction in titer to 1:2 or lower.

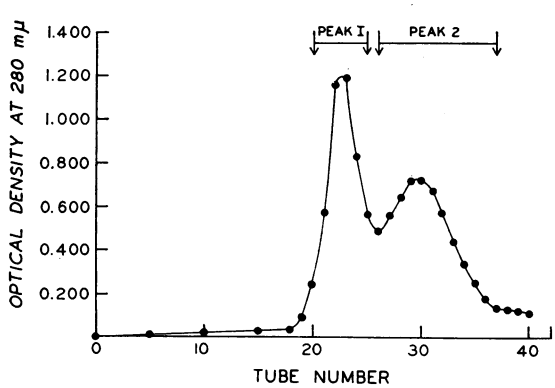

FIG. 3. ELUTION PATTERN OF REDUCED AND ALKYLATED RIP ANTI-RH γ-GLOBULIN FROM A SEPHADEX G-200 COLUMN. Three-ml fractions were collected in each tube. The arrows indicate the tubes that were pooled for subsequent studies.

Effect of pH on the saline activity of anti-Rh sera in the presence of mercaptoethanol. Preliminary experiments showed that the pH of a 1 M solution of ME in saline is 4.5. When equal volumes of the 1 M ME and protein solutions in saline were mixed, the resultant solution had a pH of 5.5. The experimental system shown in Table I was tested by using saline solutions adjusted to pH 5, 7, and 9 and a phosphate-buffered saline solution at pH 7.8. The dilutions of the serum, as well as the washing of the cells, were carried out with diluents of appropriate pH. When necessary, the pH of the ME solution was adjusted by the addition of 0.1 M NaOH or HCl. There was no detectable change in pH after the serum dilutions and ME solution were mixed. Within the ranges tested, the pH did not significantly affect the saline titers produced by ME.

TABLE I

*Effect of 0.5 M mercaptoethanol (ME) on the saline activity of various anti-Rh sera**

Anti-Rh sera†	Dilution of incomplete anti-Rh sera treated with ME									Anti-globulin titer‡
	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	Saline	
Rip anti-CD	4+	4+	4+	4+	3+	2+	Trace	0	0	8,192
4609 anti-D§	4+	4+	4+	4+	3+	3+	2+	Trace	0	8,192
4592 anti-D§	4+	4+	3+	3+	3+	2+	2+	1+	0	2,048
Pie anti-D	3+	3+	2+	1+	Trace	0	0	0	0	1,024
MS 56 anti-D	4+	4+	4+	2+	2+	Trace	0	0	0	256
Wash anti-D	3+	Trace	0	0	0	0	0	0	0	256
Ram anti-D	3+	1+	1+	0	0	0	0	0	0	128
Mal anti-D	2+	1+	1+	0	0	0	0	0	0	64
HP anti-CD	2+	Trace	0	0	0	0	0	0	0	64
Sch anti-D	1+	Trace	0	0	0	0	0	0	0	64
Bl anti-D	0	0	0	0	0	0	0	0	0	16

* The procedure used for the reduction and conversion to saline activity was that of Pirofsky and Cordova (8, 9). In all cases, O Rh-positive cells from the same donor were used. No agglutination was demonstrated when the experiments were repeated with O Rh-negative cells.

† The specificity of the various incomplete antibodies was determined by serological testing against a panel of cells of varying antigenic types. "Rip," "4609," etc., designate patients.

‡ Obtained in tests on another series of dilutions.

§ Saline titers of 1:32 and 1:64 were obtained for anti-Rh sera 4609 and 4592, respectively, in the absence of ME.

Effect of mercaptoethanol on the saline activity of purified 7 S anti-Rh γ-globulin. Since serum proteins other than γ-globulin could be responsible for the observations noted in Table I, a single anti-Rh serum (Rip anti-CD) was selected for subsequent studies. The effect of time and the concentration of ME on the "saline" activity of the γ-globulin preparation was tested by incubating equal volumes of various dilutions of the antibody γ-globulin with either 1.0 M or 0.1 M solutions of ME in saline (0.5 M or 0.05 M, respectively, final concentration) for 15, 30, and 60 minutes. At the appropriate time, one drop of a 2% suspension of O Rh-positive red cells was added to the reaction mixture. Agglutination was scored after 5 minutes of further incubation at room temperature. As shown in Table II, when a purified anti-Rh γ-globulin was used, saline activity could be produced in the presence of ME. These results confirm the observations of Pirofsky and Cordova that 0.5 M ME is more efficient than 0.05 M ME in producing saline activity within 30 minutes. In addition, as previously noted, the antiglobulin titer was not significantly affected. On the basis

TABLE II

*Effect of concentration of 2-ME and time on the saline activity of 7 S γ-globulin containing incomplete anti-Rh antibody**

Mercapto-ethanol†	Reduction period‡	Dilution of anti-Rh§								Anti-globulin titer
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	Saline	
	min									
None		0	0	0	0	0	0	0	0	1:128
0.5 M	15	2+	2+	Trace	0	0	0	0	0	1:128
	30	3+	2+	1+	Trace	0	0	0	0	1:128
	60	4+	3+	1+	1+	0	0	0	0	1:128
0.05 M	15	0	0	0	0	0	0	0	0	1:128
	30	1+	0	0	0	0	0	0	0	1:128
	60	4+	3+	2+	1+	1+	0	0	0	1:128

* The preparation was free of other contaminating serum proteins by immunoelectrophoretic and ultracentrifugal criteria.

† Final concentration during the incubation period.

‡ Time of exposure of antisera to ME. The reduction was carried out at room temperature.

§ A 1:2 dilution contains 5 mg of protein per ml. Agglutination was scored 5 minutes after the addition of erythrocytes.

of these observations, subsequent incubations with 1.0 M and 0.1 M ME were carried out for 15 and 60 minutes, respectively.

Here, as in the previous experiment, we noted that the agglutinated cells were dispersed by washing three times with saline.

Effect of reduction and alkylation of anti-Rh γ-globulin. If the saline activity of the anti-Rh in the presence of ME is a direct result of changes in the tertiary structure brought about by cleavage of disulfide bonds, then agglutination should be obtained when the reduced sulfhydryl groups of the anti-Rh molecules are blocked by alkylation. After reduction and alkylation, however, approximately 30% of the protein was aggregated (Figure 2). No aggregates could be detected by ultracentrifugation of the anti-Rh preparation in the presence of ME. Preliminary experiments showed that the reduced and alkylated protein had saline activity. Because the aggregates formed after alkylation and dialysis might in themselves be responsible for the direct agglutination, the higher molecular weight components were separated from the reduced and alkylated 7 S anti-Rh by gel filtration. The results are shown in Figure 3. Two fractions, indicated as peak 1 and peak 2, were pooled and concentrated separately.

Ultracentrifugal analysis showed that peak 1 consisted primarily of aggregates ($\backsim$ 80%) and that peak 2 consisted of reduced and alkylated 7 S protein (Figure 1). The proteins obtained from peaks 1 and 2 were tested for agglutinating activity (Table III). The protein obtained from peak 1 showed significant saline agglutination that was not affected by washing. The unaggregated protein of peak 2 had slight agglutinating activity, which may have been caused by trace amounts of aggregates not detectable from schlieren ultracentrifugation patterns. The antiglobulin titers of the unreduced 7 S protein and reduced-alkylated protein of peak 2 were not changed. The antiglobulin titer of the aggregated protein, however, was reduced to 1 : 16. This is not surprising, since the aggregates probably consisted of nonantibody as well as of antibody molecules.

Effect of mercaptoethanol treatment of cells on the saline activity of reduced and unreduced anti-Rh. According to Pirofsky and Cordova (8, 9), pretreatment of cells with ME is not a factor leading to saline activity of incomplete anti-Rh antibodies. Their experiment was repeated with some modification. The cells were treated with ME and washed with saline as described in the Methods section. Equal volumes of unreduced or reduced and alkylated 7 S protein and a 2% suspension of ME-treated or control untreated cells were mixed and scored for agglutination after 5 minutes at room temperature. As is evident from Table IV, no agglutination occurred when ME-treated or control cells were added to varying dilutions of untreated anti-Rh γ-globulin. (The end-point Coombs' titer was not affected.) The effect of the reducing agent on the cells was clearly indicated when the reduced and alkylated 7 S protein of peak 2 was tested. To determine whether the observed effects might have been attributable to a small amount of reducing agent ($< 10^{-5}$ M) carried over in the washing of the cells, ME (final concentration, 0.005 M) was intentionally added to untreated cells as a control. This amount of reducing agent did not enhance saline activity (Table IV). Although the antiglobulin titer was not affected, the agglutinates were dispersed by repeated washing. These data suggest that ME af-

TABLE III

Effect of reduction and alkylation on the saline activity of 7 S incomplete anti-Rh γ-globulin

Treatment of 7 S anti-Rh*	Dilution of anti-Rh†								End-point titer after 3 washes	Anti-globulin titer
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	Saline		
Untreated	0	0	0	0	0	0	0	0	0	1:128
Peak 1	2+	2+	1+	1+	Trace	0	0	0	1:16	1:16
Peak 2	Trace	Trace	0	0	0	0	0	0	0	1:128

* The 7 S anti-Rh γ-globulin was reduced and alkylated and passed through a column of Sephadex G-200. Peaks 1 and 2 represent the order of elution of protein from the column (Figure 2).

† A 1:2 dilution contains 5 mg of protein per ml.

TABLE IV

Saline activity of reduced and alkylated 7 S incomplete anti-Rh using O Rh-positive cells treated with ME

Anti-Rh γ-globulin	Treatment of O Rh-positive cells*	Dilution of anti-Rh†							End-point titer after 3 washes	Anti-globulin titer
		1:2	1:4	1:8	1:16	1:32	1:64	Saline		
Untreated	Saline	0	0	0	0	0	0	0	0	1:128
	Saline plus 0.005 M ME	0	0	0	0	0	0	0	0	1:128
	0.5 M ME	0	0	0	0	0	0	0	0	1:128
	0.05 M ME	0	0	0	0	0	0	0	0	1:128
Reduced and alkylated‡	Saline	Trace	Trace	0	0	0	0	0	0	1:128
	Saline plus 0.005 M ME	Trace	0	0	0	0	0	0	0	1:128
	0.5 M ME	2+	2+	2+	1+	Trace	0	0	1:2	1:128
	0.05 M ME	1+	1+	Trace	0	0	0	0	0	1:128

* The cells were incubated with 0.5 M and 0.05 M ME for 15 and 60 minutes, respectively, at room temperature, then washed with a sufficient volume of saline to give a final ME concentration of 10^{-5} M. As a control, 0.005 M ME was added to untreated cells.

† A 1:2 dilution contains 5 mg of protein per ml.

‡ The 7 S anti-Rh γ-globulin was reduced and alkylated, passed through a column of Sephadex G-200, and collected as peak 2 (Figure 3).

fects both the anti-Rh molecule and the red cell surface.

Reversal of agglutination. Since washing the agglutinates consistently caused dispersion of the cells, it appeared that ME or a nonspecific protein, or both, were necessary for saline activity. This was tested by washing the agglutinated cells with saline containing either 0.5 M ME or 1% bovine serum albumin (BSA). In each case, the titers were reduced as indicated previously. This observation suggested that both the reducing agent and protein contributed to the occurrence of agglutination. The implications of these results are discussed in the ensuing sections.

Effect of mercaptoethanol on cells sensitized with 7 S anti-Rh γ-globulin. If ME acts on the anti-Rh molecule by altering the tertiary structure, then similar results might be obtained by adding the reducing agent directly to washed, sensitized cells. Preliminary experiments showed that ME failed to elicit saline activity in such a system. This finding, together with the fact that repeated washing substantially abolished agglutination, suggested that nonantibody protein was also necessary for saline activity. This hypothesis was tested as follows: O Rh-positive cells were sensitized with varying amounts of anti-Rh γ-globulin for 1 hour at 37° C. One drop of a 2% suspension of the washed, sensitized cells was added to an equal volume of a purified 7 S human γ-globulin preparation that had been prereduced at room temperature with 0.05 M or 0.5 M ME for 15 and 60 minutes, respectively; the γ-globulin preparation had no agglutinating or incomplete anti-Rh activity. Agglutination was scored after the mixture had been incubated for 5 minutes at room temperature (Table V). No agglutination occurred when ME was added directly to the sensitized cells. Agglutination was obtained, however, when varying amounts of the prereduced nonspecific human γ-globulin were added in the presence of reducing agent. The degree of agglutination appeared to be dependent both on the concentration of protein and the amount of anti-Rh used for sensitization. At the lower concentration of reducing agent, agglutination was also observed, but to a substantially lesser degree.

Agglutination was not demonstrable when a 1:2 dilution of protein was added in the absence of ME (Table V). This dilution of γ-globulin contains 5 mg of protein per ml, which is one-sixtieth the amount of protein, BSA, used routinely to detect incomplete Rh antibodies. An antiglobulin end-point titer of 1:128 confirmed that the cells were sensitized.

The results of similar tests with a purified γ_{1A}-myeloma protein and a γ_{1M}-macroglobulinemia

TABLE V

*Effect of concentration of ME and a nonspecific protein on the saline activity of anti-Rh sensitized erythrocytes**

Mercapto-ethanol	Dilutions of nonantibody γ-globulin†	Direct agglutination of cells sensitized with varying dilutions of 7 S anti-Rh‡								
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Saline
0.5 M	Saline	2+	0	0	0	0	0	0	0	0
	1:8	4+	3+	3+	3+	3+	2+	1+	0	0
	1:64	2+	2+	2+	1+	Trace	Trace	0	0	0
	1:512	Trace	0	0	0	0	0	0	0	0
0.05 M	Saline	0	0	0	0	0	0	0	0	0
	1:8	1+	1+	Trace	Trace	0	0	0	0	0
	1:64	Trace	Trace	0	0	0	0	0	0	0
	1:512	0	0	0	0	0	0	0	0	0
Saline§	1:2	0	0	0	0	0	0	0	0	0

* The sequence of the experiment was as follows: Equal volumes of nonspecific protein were incubated with ME for 15 or 60 minutes; sensitized cells were then added. After standing for 5 minutes, the mixtures were scored for agglutination.

† A 1:2 dilution contains 5 mg of protein per ml. No antierythrocyte activity could be detected by either direct agglutination or the antiglobulin reaction.

‡ The cells were sensitized by mixing serial twofold dilutions of the anti-Rh preparations with equal volumes of a 2% red cell suspension. The cells were then allowed to incubate at 37° C for 1 hour, washed three times with saline, and reconstituted to a 2% suspension in saline.

§ The antiglobulin end-point titer was 1:128.

protein were comparable to those reported in Table VI.

Effect of other nonspecific proteins on the saline activity of sensitized cells in the presence of mercaptoethanol. The effect of proteins of nonhuman origin, bovine γ-globulin (BGG), and ovalbumin (EA) on the ability to elicit saline activity of cells sensitized with anti-Rh was studied. The results are given in Table VI. Mercaptoethanol-induced saline activity was observed only when protein was present, and the degree of agglutination appeared to be dependent on the protein concentration. The

TABLE VI

*Effect of reduction of ovalbumin (EA) and of bovine γ-globulin (BGG) on the saline activity of anti-Rh sensitized erythrocytes in the presence of 0.5 M ME**

	Dilution of protein†	Direct agglutination of cells sensitized with varying dilutions of 7 S anti-Rh‡							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	Saline
EA plus 0.5 M ME	Saline	0	0	0	0	0	0	0	0
	1:8	2+	2+	4+	3+	3+	2+	0	0
	1:64	2+	1+	1+	0	0	0	0	0
	1:512	0	0	0	0	0	0	0	0
BGG plus 0.5 M ME	Saline	Trace	0	0	0	0	0	0	0
	1:4	3+	3+	3+	2+	2+	2+	Trace	0
	1:16	2+	2+	1+	1+	Trace	0	0	0
	1:64	1+	Trace	0	0	0	0	0	0
EA or BGG plus saline§	1:2	0	0	0	0	0	0	0	0

* The sequence of the experiment was as follows: Equal volumes of EA or BGG were incubated with 1.0 M ME for 15 minutes; sensitized cells were then added. After standing for 5 minutes, the mixtures were scored for agglutination.

† A 1:2 dilution contains 5 mg of protein per ml. No antierythrocyte activity could be detected by either direct agglutination or the antiglobulin reaction.

‡ The cells were sensitized by mixing serial twofold dilutions of the anti-Rh preparations with equal volumes of a 2% red cell suspension. The cells were then allowed to incubate at 37° C for 1 hour, washed three times with saline, and reconstituted to a 2% suspension in saline.

§ The antiglobulin end-point titer was 1:128.

ability of BGG and EA to elicit saline activity was somewhat less than that observed for a nonspecific human γ-globulin, but the titers observed were sufficiently high to be significant. These results suggest that any protein will support agglutination of cells sensitized with incomplete antibody in the presence of ME.

Discussion

Our data confirm and extend the reported observations (8, 9) that sera containing "incomplete" anti-Rh antibodies can produce agglutination of O Rh-positive cells but not of O Rh-negative cells in the presence of ME. They also demonstrate that γ_G-globulin preparations from such sera also produce direct agglutination in the presence of ME. Anti-CD and anti-D antibodies behaved similarly with respect to ME-induced agglutination of red cells from the same donor, as well as red cells of varying phenotype (R_1R_1, R_2R_2, and R_0). No difference in titer against cells of one or another phenotype was detectable with the two-fold dilution method employed (14). This is of interest since red cells of different phenotypes may vary significantly in their capacity to bind and retain anti-Rh antibodies (15).

Pirofsky and Cordova (8, 9), on the basis of this ME-induced agglutination, postulated that incomplete anti-D is a bivalent antibody. They suggested that only one of the combining sites of the incomplete antibody is available for interaction with the red cell antigen and that alteration of the tertiary structure of the antibody molecule by ME exposes the previously hidden site, making it available for agglutination. If this phenomenon exists, reduction and alkylation should result in the same change in tertiary structure of the incomplete antibody molecule, which could then agglutinate red cells in the absence of ME. In our experiments, reduced and alkylated anti-Rh antibody, free of aggregates, was unable to agglutinate the appropriate red cells (Table III) unless the cells were also pretreated with ME (Table IV). These results indicate that agglutination is brought about by changes exerted by ME on both the cells and the antibody.

The 7 S anti-D preparations used in this study, although immunologically pure γ_G-globulin, were not specifically purified antibody, and hence consisted mainly of γ-globulin without anti-D specificity. Consequently, the participation of non-anti-D γ_G material in the agglutination induced by ME must be considered. The fact that cells sensitized with anti-Rh antibody, washed, and then incubated with ME were not agglutinated suggests that the nonantibody γ-globulin contributes in some way to ME-induced agglutination. When this hypothesis was tested by incubating 7 S human γ-globulin without anti-Rh activity with ME and then adding Rh-positive cells sensitized with incomplete anti-D, agglutination did take place. Such agglutination proved to be dependent both on the amount of nonantibody protein in the reaction mixture and on the degree of sensitization by the anti-Rh antibody. Subsequent studies showed that a variety of proteins from heterologous sources could also effect agglutination of Rh-sensitized cells in the presence of ME. Microscopic observations confirmed that these results were not due simply to the effect of protein concentration (rouleaux); furthermore, the concentrations of protein used were much lower than the 30% albumin routinely employed to produce agglutination of incomplete antibody. In addition, ME was required for agglutination to take place.

In all experiments where agglutination occurred, washing the agglutinated cells with saline resulted in a substantial reduction in titer. This observation suggests that agglutinations produced by ME and by 30% BSA are analogous in their nonimmunological nature, in that the agglutinated cells in both cases can be dispersed by washing.

Several factors appear to be involved in the ME-induced agglutination of O Rh-positive cells by incomplete anti-Rh antibody. These include *1*) the effect of ME on antibody and nonantibody protein in the reaction mixture, *2*) the effect of ME on the red cell, and *3*) the sensitization of the red cells by incomplete antibody. The information obtained in this study permits speculation on the influence of these factors in ME-induced agglutination. The reaction between red cell antigens and isoantibodies presumably occurs in two stages. The first stage, a specific immunochemical reaction, involves binding of antibody to antigen on the red cell stroma. With ME-induced agglutination, the second stage of the isoantibody-antigen reaction, agglutination of the red cell mass may perhaps be a nonspecific process influenced by the suspending

medium or by alteration of the red cell surface.[9] The latter suggestion is supported by the observation of Davis, Green, and Tymms (16), in studies by electron microscopy, that changes on the red cell surface may be brought about by sensitization with anti-D. In addition, it is generally agreed that enzymes, by altering the red cell surface, enhance agglutination of sensitized cells. Similar changes may well occur when the red cell is treated with ME; this point is presently under investigation.

Agglutination of cells sensitized with incomplete anti-Rh can be achieved by the use of large anisometric molecules in the suspending medium (17), by enzymatic digestion of red cell stroma (18, 19), and by the antiglobulin reaction (20). Jandl and Simmons (21) showed that multivalent metallic cations can attach to red cell membranes in sufficient quantity to produce agglutination. They suggested that these metallic cations effectively reduce the negative net charge on the red cell membrane, thus enhancing agglutination. This effect does not seem to be analogous to that of ME. It is unlikely that ME would have sufficient effect on the charge of the red cell membrane to cause agglutination, since treatment with ME alone does not cause red cell agglutination.

Among the many effects of reducing agents on proteins, the least well known is that of protein-protein interaction. Our experiments demonstrate that after reduction and alkylation, a portion of the molecules undergoes denaturation and subsequent aggregation (Figure 2), which may or may not be sulfhydryl activated. It is possible that some of the molecules may also possess an unusual "adhesiveness" toward other molecules. This adhesive effect cannot be measured by physical-chemical techniques such as ultracentrifugation (22), viscosity (22), or optical rotation (23). When washed, sensitized cells are placed in a medium of ME and protein, however, adhesiveness is manifested by the reversible agglutination of the erythrocytes. The fact that washed, sensitized cells are not agglutinated by ME alone can be attributed to the absence of sufficient adhesive protein in the medium to cross-link the cells.

A schematic illustration of the possible reaction sequence when sensitized cells are exposed to ME and nonspecific protein is shown in Figure 4. Sensitized cells, either in the presence or absence of ME, do not agglutinate. If the medium contains sufficient adhesive protein, agglutination does take place. Although the effect of ME on the erythrocyte surface is not known, disulfide bonds may be split, as shown in Figure 4, resulting in a geometric change in the red cell membrane. The consequent change in orientation of the antibody molecule on the red cell surface would make a larger portion of the molecule available for interaction with other adhesive molecules.

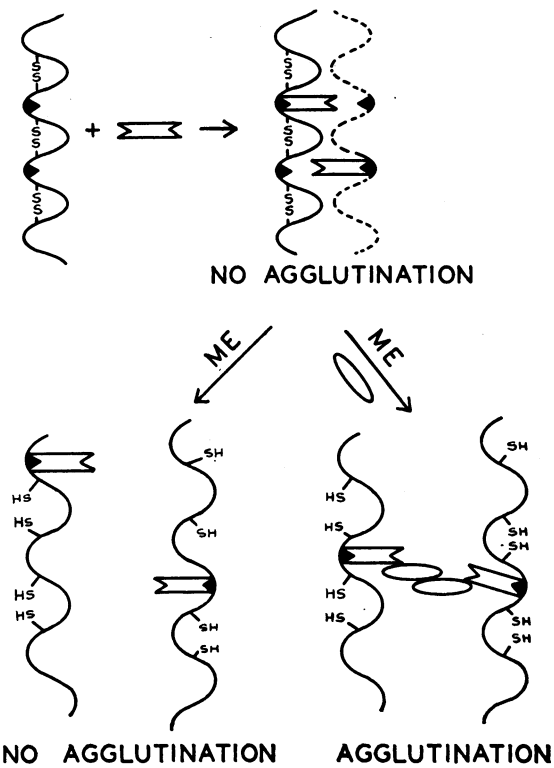

FIG. 4. SCHEMATIC ILLUSTRATION OF THE EFFECT OF MERCAPTOETHANOL (ME) ON THE SALINE ACTIVITY OF INCOMPLETE ANTI-RH MOLECULE.

Mercaptoethanol treatment does not appear to be as sensitive as the antiglobulin reaction in detecting the presence of incomplete or blocking antibody. In our experiments ME-induced agglutination occurred only with anti-Rh sera of high titer. The possibility remains that treatment of red cells with ME may expose previously blocked antigenic sites, which would explain such diverse phenomena as the weakly reactive Rh cells (24),

[9] Evidence that the second stage of agglutination is not nonspecific, at least with respect to agglutination of enzyme-treated cells, has been provided by Prager (5).

the -D- cells (25, 26), and the - - - (deletion) cells (27). Investigation of this and other possibilities is currently in progress in our laboratory.

Summary

The direct agglutination of Rh-positive erythrocytes produced by the simultaneous addition of mercaptoethanol and "incomplete" anti-Rh antibody appears to be produced by mechanisms other than changes in the tertiary structure of the molecules. Data obtained in experiments with isolated 7 S γ-globulin from anti-Rh sera and mercaptoethanol-treated red cells suggest that several processes are involved. These include the direct effect of mercaptoethanol on the red cell surface, mercaptoethanol-induced "adhesiveness" of protein molecules, and sensitization of erythrocytes by incomplete antibody. The serological behavior of "incomplete" antibodies in the presence of mercaptoethanol appears to be attributable to a nonspecific, nonimmunological phenomenon.

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