The Role of Antigen Mobility In Anti-Rh$_o$(D)-Induced Agglutination

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ABSTRACT Intact human erythrocytes were cross-linked with glutaraldehyde (GA) or dimethyladipimidate (DMA) and tested for their ability to bind [^{125}I]-IgG anti-Rho(D) and to undergo antibody-mediated hemagglutination. There was no decrease in antibody binding after treatment with GA concentrations up to 1.25% and DMA concentrations up to 1%. Red cells treated with these concentrations of GA and DMA did not agglutinate. The techniques employed to induce agglutination of the cross-linked red cells involved "incomplete" IgG anti-Rho(D) in albumin, "complete" IgM anti-D Rho(D) in saline, and the antiglobulin (Coombs) reaction. The agglutinability of the chemically modified red cells was inversely correlated with the extent of fixation. The dissociation of antibody binding from agglutinability in cross-linked erythrocytes suggests that Rho(D) antigen mobility is required for red cell agglutination. Antigen mobility was manifested by the transition from a relatively monodisperse distribution pattern of Rho(D) antigen sites to one of large aggregates or clusters when agglutination was induced by IgM anti-Rho(D), IgG anti-Rho(D) agglutination of protease modified red cells, and by anti-IgG agglutination of IgG anti-Rho(D)-coated red cells. Antigen clustering was not as prominent in red cells agglutinated by IgG anti-Rho(D) in the presence of albumin. Even though antigen mobility is a prerequisite for antibody-mediated hemagglutination, clustering does not appear to be an absolute requirement. The degree of antigen clustering differs with varying types of agglutination.

INTRODUCTION

Antibody-induced red cell agglutination is a complex phenomenon of general interest to cell biology and of clinical relevance in establishing compatibility for blood transfusion. Analysis of the hemagglutination reaction in the past has emphasized the role of immunoglobulin class (40, 50), the effect of quantity of cell-bound antibody (15, 41), and the role of electrochemical effects both with respect to antibody binding and to the subsequent agglutination of antibody-coated cells (38). More recently, however, evidence has been accumulating that suggests that freedom of translational mobility for membrane components is a fundamental property of cell surfaces (9, 45). Red cell agglutination by interconnecting agglutinins can thus be viewed as requiring the aggregation or clustering of enough cell surface receptors on each cell to overcome the electrostatic forces of intercellular repulsion (34).

The Rho(D) antigen is a lipid-dependent membrane protein (10, 11), which ultrastructurally displays an aperiodic, relatively monodisperse distribution throughout the membrane (32). Unlike the carbohydrate-bearing ABO antigens and those cell surface receptors studied with lectins, the dynamic behavior of the Rho(D) antigen may be indicative of nonglycoprotein components of the red cell membrane. We have investigated the ultrastructural distribution of the Rho(D) antigen under agglutinating conditions and the effect of cross-linking on antibody binding and agglutinability of intact red cells. Our results suggest that Rho(D) antigen mobility is required for hemagglutination and that differing degrees of antigen clustering accompany hemagglutination, depending on the mechanism used to induce agglutination. A preliminary account has appeared (51).

METHODS

The preparation of [^{125}I]labeled anti-Rh$_o$(D) has been described (28).

Glutaraldehyde (GA)[1] *treatment of red cells.* Freshly drawn blood in Alsever's solution was washed twice with 1:10 phosphate-buffered normal saline (BNS), pH 6.5, and the cells were suspended at about 2% hematocrit in BNS. An equal volume of the desired concentration of neutralized GA in BNS was then added and the cells were incubated for 5 min at room temperature. After the addition

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[1] *Abbreviations used in this paper:* BNS, phosphate-buffered normal saline; BSA, bovine serum albumin; Con A, concanavalin A; DMA, dimethyladipimidate; Fer-HGG, ferritin-conjugated rabbit anti-human IgA; GA, glutaraldehyde.

of one-half vol of 5% bovine serum albumin (BSA), made up in BNS, the cells were centrifuged for 90 s, at 2,400 *g*. After a wash with 0.5% BSA in BNS, the cells were suspended in BNS and their concentration was determined with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Dimethyladipimidate (DMA) treatment of red cells. Freshly drawn blood in Alsever's solution was washed three times with borate-buffered saline, pH 8.5. After adjusting the final pH to 8.5 with 0.2 N NaOH, the cells were adjusted to about a 40% hematocrit. DMA solutions were prepared by dissolving solid DMA in a minimal volume of 5 N NaOH, and then diluting with borate-buffered saline. The final pH was adjusted to 8.5 when necessary. DMA solutions and erythrocytes at pH 8.5 were mixed at a 1:1 volume ratio and incubated for 2 h at 37°C. After several BNS washings, the red cell suspension was counted and used experimentally.

The most striking and recognizable effect of GA or DMA treatment of erythrocytes was the development of resistance to hypotonic lysis. The extent of cross-linking could be readily followed by measuring hemoglobin release under hypotonic stress. In addition, cross-linked cells changed from the native biconcave disk to a more spherical shape. As a consequence, the packed cell volume of modified cells was greater than that for the untreated cells. This was reflected in a lower cell count for the fixed cells when compared to untreated cells of identical hematocrit value.

Agglutination reactions. Agglutination was carried out with two types of anti-Rh_o(D) typing sera: a saline-active "complete" IgM antiserum and an albumin-enhanced, "incomplete" IgG antiserum. Cells sensitized with IgG anti-Rh_o(D) were agglutinated with antiglobin serum. The procedures followed were essentially as described in the literature provided by the manufacturer (Ortho Pharmaceutical Company, Inc., Raritan, N. J.). Agglutination of papain-modified red cells by IgG anti-Rh_o(D) was carried out as described previously (26). Papain-modified Rh_o(D) positive red cells undergo direct agglutination (3+ to 4+) after incubation with IgG anti-Rh_o(D) and bind at equilibrium 30–80% more antibody than the untreated, unagglutinated red cells (16, 26). The extent of agglutination (0 to 4+) was scored macroscopically by conventional serological techniques and on occasion checked microscopically by phase contrast light microscopy.

[^{125}I]Anti-Rh_o(D) binding by intact and fixed erythrocytes. The standard incubation mixture consisted of 0.05 ml red cells from a 10% hematocrit suspension and varying volumes of labeled antibody. After a 1-h incubation at 37°C, the sensitized red cells were washed four times with an excess of cold BNS, pH 6.5, containing 0.3 BSA, and the amount of cell-bound ^{125}I was determined in a well-type scintillation gamma counter. Results are expressed as micrograms N bound per 10^{10} red blood cells, calculated as described previously (27).

Immunoelectron microscopy. Ferritin-conjugated rabbit anti-human IgG (Fer-HGG) was prepared with toluene-2,4 diisocyanate as a coupling agent. The ferritin conjugate was separated from unconjugated ferritin and free IgG by agarose column chromatography (32). The Fer-HGG fractions used for staining contained from 2 to 5 mg protein/ml and had titers that ranged from 40 to 180 when tested against R_1r red cells sensitized with a 1:5 dilution of a high titered anti-Rh_o(D) serum.

Red cells sensitized with both unlabeled and labeled IgG anti-Rh_o(D) were lysed at an air-water interface and the floating membranes picked up from above on carbon-strengthened, collodion-coated electron microscopy grids, as described by Nicolson, Masouredis, and Singer (32). In studies involving commercial IgM anti-Rh_o(D), the presence of contaminating IgG (14) permitted the use of Fer-HGG to visualize the ultrastructural pattern of the Rh_o(D) antigen sites that bound IgG anti-Rh_o(D). After ferritin-conjugate staining of the ghost surface not adherent to the grid, the specimens were examined in a Zeiss Model 9S microscope (Carl Zeiss, Inc., New York).

Chemicals. Ultrapure GA was obtained in small ampoules as a 50% solution from Tousimis Research Corp. (Rockville, Md.). The quality of the GA reagent was very important. Unless freshly distilled reagent or ultrapure grade GA was used, the amount of labeled anti-Rh_o(D) that could be bound by treated red cells was markedly decreased. Furthermore, the concentration of GA necessary to inhibit agglutination to a given score value was reduced by as much as 10-fold. A possible explanation is that aged solutions (over 1 day) contain oligomeric GA (23) which, although very effective as a cross-linker, may also produce inactivation or masking of Rh_o(D) antigen sites. DMA was purchased from Pierce Chemical Co. (Rockford, Ill.). Papain was obtained from Matheson Coleman & Bell (Norwood, Ohio), and used without further purification.

RESULTS

Figs. 1 and 2 represent results obtained in 10 and 4 experiments with GA and DMA, respectively. There were no qualitative differences among red cells of different antigen phenotypes nor with two labeled antibody preparations.

Effect of GA and DMA cross-linking on anti-Rh_o(D) binding. Neither GA nor DMA pretreatment with concentrations less than 1.25% decreased the binding of labeled antibody to treated red cells (Figs. 1*a* and 2*a*). Under certain conditions, however, there was a significant increase in quantity of antibody bound to GA-treated cells. Cells pretreated with 0.125% GA showed an unexplained 20–30% increase in antibody binding when incubated with saturating antibody concentrations. It is not clear if this is due to a shift in the binding equilibrium of the antigen-antibody reaction or to the exposure of cryptic antigen sites. In all other instances the amount of antibody bound by either GA- or DMA-modified cells did not differ by more than 15% from that bound to untreated cells.

Effect of GA and DMA cross-linking on anti-Rh_o(D)-induced agglutination. Fixation by GA or DMA rendered erythrocytes less susceptible to direct agglutination by incomplete (IgG) or complete (IgM) anti-Rho(D) sera (Figs. 1*b*, 1*c*, and 2*b*, 2*c*). Agglutination was abolished by 0.125% GA and 1% DMA pretreatment, at which concentrations there was no loss of antibody-binding activity.

There was a similar reduction in agglutinability of GA- and DMA-treated erythrocytes in indirect agglutination of [^{125}I]anti-Rho(D)-coated red cells by anti-human IgG (antiglobulin reaction). The agglutination scores of untreated and of GA- and DMA-treated red cells having approximately the same quantity of cell-

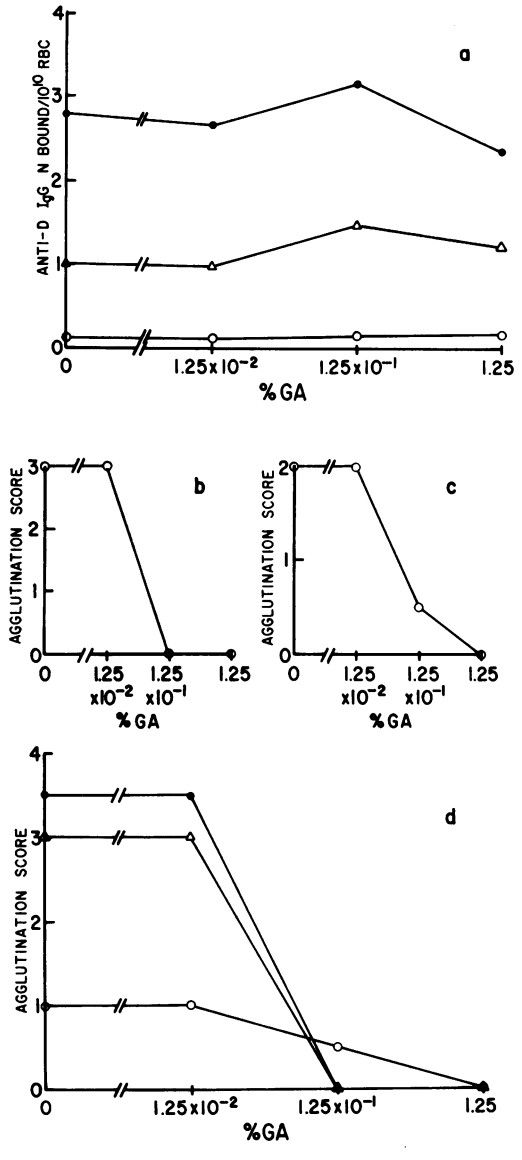


FIGURE 1 GA effects and anti-Rh_o(D) hemagglutination. *a*. Effect on anti-Rh_o(D) antibody binding. The results shown were obtained with Rh-positive O, R_1r cells and increasing concentrations of [^{125}I]IgG anti-Rh_o(D) preparation A 132 (0.207 μg N/ml) under standard incubation conditions. ○, △, and □ represent incubations with 0.01, 0.1, and 0.7 ml, respectively, of labeled antibody solution. Rh-negative cells, intact or cross-linked, bound less than 6.8% of control values. *b*. Effect on direct agglutination by anti-Rh_o(D) serum containing IgG antibodies. Experiments with 0, R_1r cells were as described in Methods. Rh-negative cells, intact or cross-linked, did not agglutinate. *c*. Effect on direct agglutination by anti-Rh_o(D) serum containing IgM antibodies. Same conditions as 1*b*. *d*. Effect on indirect agglutination. Rh-positive, 0, R_1r cells were coated with varying levels of labeled antibody A 132 and agglutinated with antiglobulin serum as described in the text. ○, △, and □ represent cells having an average antibody content of 0.148, 1.185, and 2.747 μg N/10^{10} cells. Rh-negative cells, fixed or unfixed, did not agglutinate.

bound antibody are shown in Figs. 1*d* and 2*d*. Agglutination was abolished with 0.125% GA and 1% DMA, the same range as in direct agglutination.

Immunoelectron microscopy. The ultrastructural distribution of cell-bound IgG anti-Rho(D) (i.e. Rho(D) antigen receptors) on agglutinated and unagglutinated red cells is shown in Figs. 3 and 4. The figures contain representative micrographs that contrast the ferritin particle distribution on unagglutinated anti-Rho(D) IgG-coated red cells with that observed after agglutination. Hemagglutination of antibody-coated cells was induced by commercial IgG anti-Rho(D) agglutinating serum in albumin, papain treatment, and a commercial IgM anti-Rho(D) agglutinating serum in saline. With the exception of IgG anti-Rho(D) in albumin, all agglutinating reagents led to extensive redistribution of ferritin particles into large discrete masses containing up to about 50 ferritin particles/cluster.

Fig. 3*b* shows the distribution of ferritin particles (average 756/μm^2) on Rh-positive (R_1R_1) red cells agglutinated with commercial, saline-active, IgM anti-Rho(D). The saline-suspended red cells after incubation with the IgM antibody showed a 1+ macroscopic agglutination upon centrifugtion. The ferritin-stained receptors represent the distribution of Rho(D) antigen sites occupied by IgG anti-Rho(D), which constitutes some 20–30% of the total anti-Rho(D) content of commercial IgM-typing sera (14). Rho(D) receptors containing IgM antibody are not visualized with the anti-IgG conjugate.

Fig. 3*a* shows the distribution of ferritin particles (average of 328/μm^2) on the same Rh-positive red cell sensitized with [^{125}I]anti-Rho(D) IgG. There was an average of 16,000 Rho(D) antigen sites/red cell, as calculated from the quantity of radioactivity bound to the red cells. No agglutination was evident upon macroscopic observation.

It is visually apparent from the two micrographs that there is significantly more aggregation of the ferritin particles in the red cells agglutinated by the IgM antiserum as compared to the unagglutinated IgG-sensitized red cells. The ferritin distribution pattern in the unagglutinated IgG-sensitized red cells shows a predominance of isolated ferritin clusters that contain less than eight particles per cluster, whereas there is a redistribution of the ferritin into large aggregates that contain in excess of 20 or more ferritin particles in the IgM-agglutinated cells.

Fig. 3*d* shows the ferritin particle distribution (average of 359/μm^2) in Rho(D)-positive (R_1R_1) red cells agglutinated by the microscope slide technique with a commercial IgG incomplete anti-Rho(D) and albumin enhancement. There was 3+ macroscopic agglutination of the red cells. The distribution of ferritin in these ag-

glutinated cells does not differ significantly from that found on unagglutinated IgG anti-Rho(D)-sensitized cells (Fig. 3*a*).

Fig. 3*c* shows the distribution of ferritin particles observed when the IgG anti-Rho(D)-sensitized cells shown in Fig. 3*a* are treated with 1% GA for 2 min on the copper grid before being stained with the ferritin anti-IgG. Significantly less aggregation of ferritin particles is observed when the anti-Rho(D)-coated cell membranes are cross-linked with GA before staining. There is also a noticeable drop in ferritin particle density (average 236/μm^2) after GA treatment.

Fig. 4*b* displays the distribution of ferritin on Rh-positive (R_1R_1) red cells treated with papain before sensitizing with [^{125}I]anti-IgG Rho(D). Strong agglutination, 3+ macroscopic, ensued after incubation with the labeled anti-Rho(D). Marked aggregation of ferritin particles into large clusters is evident, as contrasted to the untreated anti-Rho(D)-sensitized control cell shown in Fig. 4*a*. The number of D antigen sites per red cell as determined from the bound radioactivity increased from 9,300 on the untreated red cell to 12,400 on the enzyme-modified red cell. Direct counting of ferritin particles per cluster confirms the visual impression of clustering in the papain-modified agglutinated cells: 53% of the clusters contained more than 10 ferritin particles, whereas only 16% had more than 10 ferritin particles/cluster in the unagglutinated cells.

Fig. 4*c* shows the ferritin particle distribution on an Rh positive (R_1R_1) red cell incubated with BSA to evaluate nonspecific staining by the conjugate. There were less than 700 ferritin particles/cell.

Fig. 4*d* displays the ferritin particle distribution on an Rh-negative (*rr*) red cell stained with the ferritin conjugate after incubation with the [^{125}I]IgG anti-Rho-(D) to evaluate the Rh specificity of the conjugate staining. The background ferritin particle value was less than 600/cell.

DISCUSSION

Our results show that the potential for redistribution of Rho(D) antigen sites and their topological arrangement play an important role in antibody-induced Rh hemagglutination. Stabilization of red cell membrane components by cross-linking reagents (GA and DMA) strongly inhibits or abolishes Rh agglutination, whether induced directly by IgG, or IgM anti-Rho(D), or indirectly by anti-IgG, without significantly affecting antibody uptake. Depending on the means used to induce agglutination, there was extensive or no redistribution of Rho(D) antigen sites into large aggregates on the unfixed agglutinated red cells. These findings support the conclusion that Rho(D) antigen mobility is a prerequisite for agglutination.

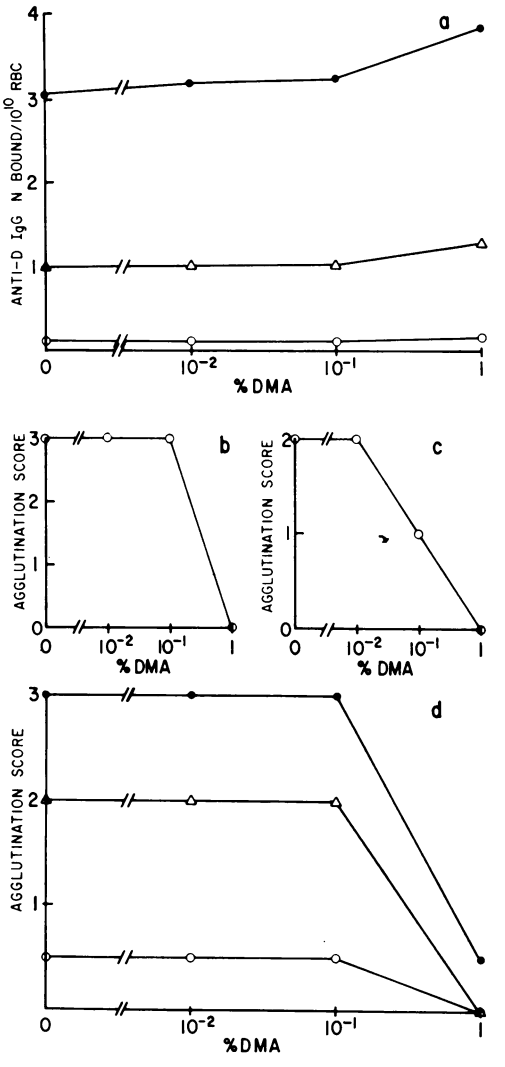
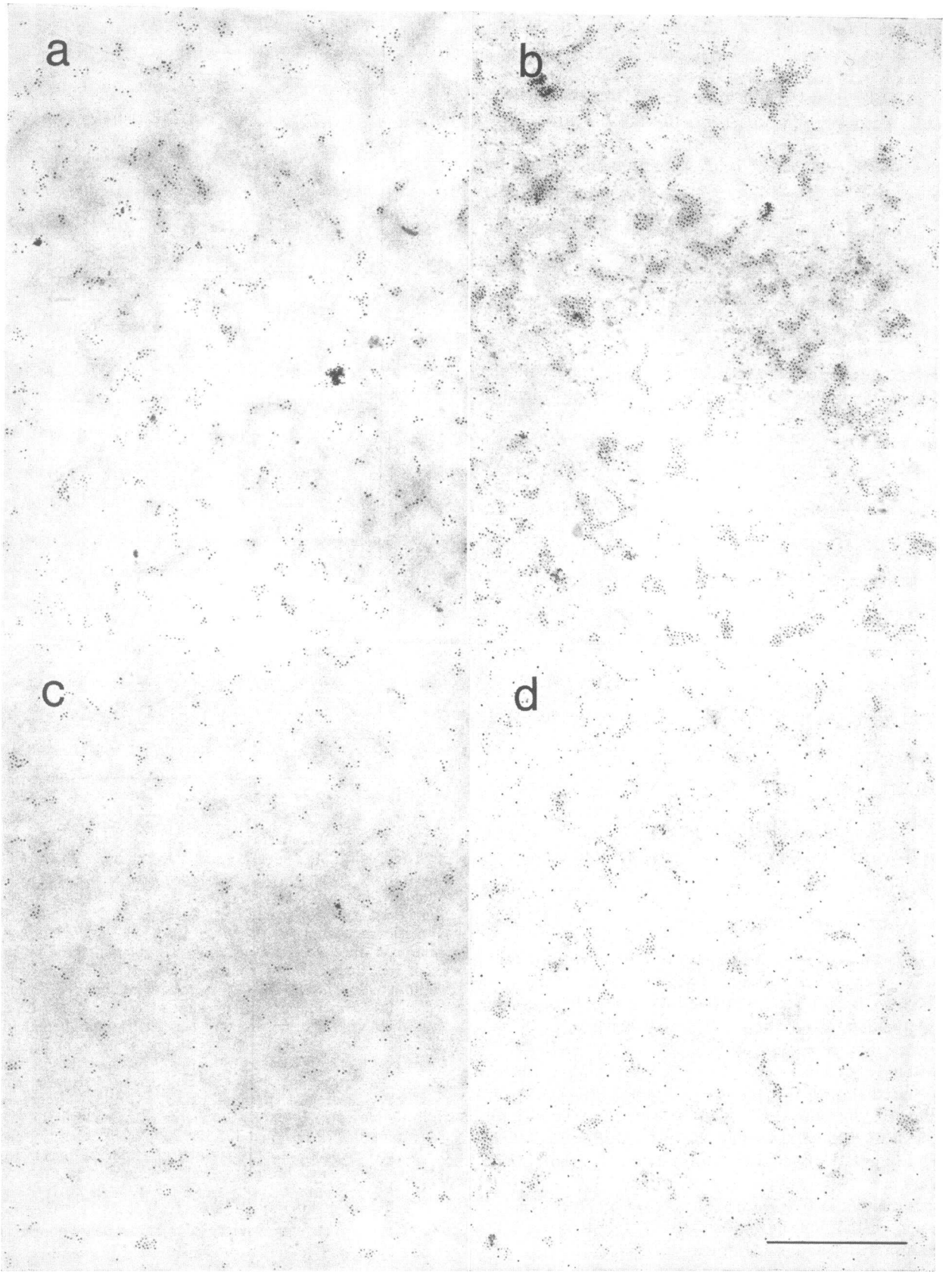


FIGURE 2 DMA effects and anti-Rh_o(D) hemagglutination. *a*. Effect on anti-Rh_o(D) antibody binding. The results shown were obtained with a Rh-positive 0, R_1r, incubated with [^{125}I]anti-Rh_o(D) preparation A 132 (0.207 μg N/ml). Standard incubation conditions were used. ○, △, and □ represent incubations with 0.01, 0.1, and 0.7 ml of antibody solution, respectively. Rh-negative cells bound, fixed or unfixed, less than 2.7% of control values. *b*. Effect on direct agglutination by anti-Rh_o(D) serum containing IgG antibodies. Experimental conditions same as in Fig. 1*b*. *c*. Effect on direct agglutination by anti-Rh_o(D) serum containing IgM antibodies. Experimental conditions same as in Fig. 1*c*. *d*. Effect on indirect agglutination. Antiglobulin serum-induced agglutination of Rh-positive, 0, R_1r cells, was carried out as described in the text. ○, △, and □ represent antibody-coated cells having an average labeled anti-Rh_o(D) content of 0.121, 1.077, and 3.309 μg N/10^{10} cells, respectively. Experimental conditions are given in Methods. Rh-negative cells, fixed or unfixed, did not agglutinate.

GA has been employed extensively to study the mobility-dependence of a variety of membrane phenomena. It can block pH-induced movement of intramembranous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particles in red cells (37); glycerol-induced aggregation of intramembranous particles in lymphoid cells (29); temperature-induced alveolar membrane particle aggregation in *Tetrahymena* (46); rotational and translational motion in rhodopsin (1, 39); "capping" of concanavalin A (con A) receptors in murine lymphocyte, fibroblast, and neuroblastoma cells (4, 5); redistribution of con A binding sites in 3T3 murine fibroblasts (3); and conA-induced agglutination of lymphoma cells (19, 42). Except for one study involving GA (44), it does not appear that GA or DMA (31) fixation, per se, can lead to redistribution of membrane components.

DMA is one of a family of bifunctional reagents, the diimidoesters, specific for primary amino groups (18). Since imidoesters retain charged amido groups ($= + NH_2$) after reacting, the amidination of the red cell membrane would not be expected to produce a net change in the frequency of charged groups on the cell surface (24, 35). GA also acts predominantly on primary amino groups. Because GA is uncharged, the interaction of GA with erythrocytes should result in a decreased number of membrane positively charged groups. It is unlikely, however, that the decreased agglutinability of GA-treated red cells is due to significant changes in the net charge of the treated red cell (13). The similarity of results on Rh agglutination with both GA and DMA, in spite of their different effects on red cell charge, indicates that charge alteration is not the primary effect of these reagents. Furthermore, at physiological pH and ionic strength, the net charge of red cells is overwhelmingly negative, with no detectable contributions by electropositive groups (30, 43). In addition, recent work (49) suggests that, contrary to earlier studies (38), it may not be justifiable to attribute a primary role to the red cell zeta potential in Rh agglutination. Another possible explanation, which cannot be ruled out, is that the decreased agglutinability of GA- and DMA-treated red cells is due to changes in the mechanical properties of the cross-linked red cells. Such cells show a general increase in cell rigidity, loss of membrane plasticity, and disk-to-sphere transformation. A more likely explanation, supported by the ultrastructural evidence for antigen clustering, is that GA and DMA treatment decreases agglutinability by restricting antigen mobility in the plane of the membrane.

Spin-label studies on GA-treated membranes indicate that GA acts by restricting the diffusional mobility of membrane proteins while leaving lipid mobility relatively unaffected (22). Moreover, GA apparently selectively cross-links the nonglycoprotein components of the human erythrocyte membrane (2, 48). DMA also acts predominantly on membrane proteins (25), but cross-links only the glycoproteins present in the membrane (20). Thus, GA and DMA, in a sense, are complementary reagents that act on different types of membrane proteins. That both inhibit agglutination suggests that restricting the mobility of membrane glycoproteins indirectly restricts nonglycoprotein mobility, and vice versa (13).

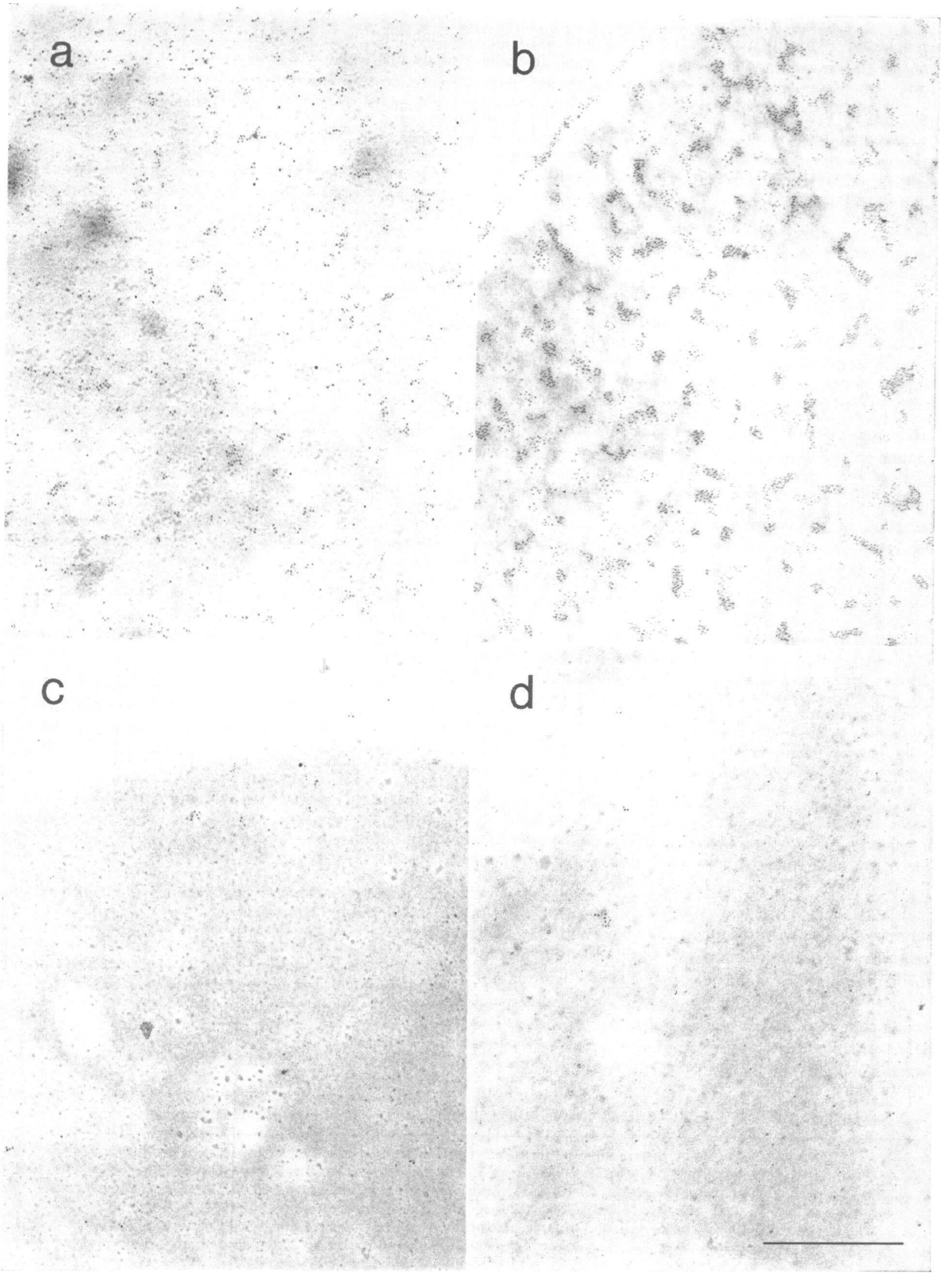
The results obtained on the effect of cross-linking reagents on red cell agglutination are supported by the ultrastructural studies on Rho(D) antigen site distribution patterns. Red cell agglutination was associated with extensive Rho(D) antigen clustering when agglutination was produced by IgM anti-Rho(D) and IgG anti-Rho(D) acting on papain-modified red cells. It is also apparent that antigen clustering can be produced by ferritin anti-IgG as the IgG-coated red cell membranes are being stained by the conjugate on the copper grid (6, 47). The degree and extent of antigen clustering produced by the ferritin conjugate probably depends on the titer of the conjugate and staining time as well as antigen site density.[2] In an earlier study (32), Rho(D)-positive red cells sensitized with [^{125}I]IgG anti-Rho(D) did not reveal significant degrees of antigen clustering. It is apparent now that this was due, at least in part, to the use of low-titered ferritin conjugates.

Not surprisingly, the conjugate-induced clustering can be blocked by pretreating the red cells with GA before conjugate staining (Fig. 3*a* and *c*). It seems unlikely that GA inactivates conjugate-binding sites on cell-bound IgG since there are for each IgG more than 100 molecules of membrane proteins to interact with the GA.

A distinction should be made between antigen clustering (reflected in aggregation of ferritin) and ferritin particle density. There is no direct relationship between the total number of ferritin particles and the number of cell-bound IgG molecules because the IgG: anti-IgG combining ratio can vary from 1 to 7 or 8. Evidence presented elsewhere[2] indicates that in the presence of antigen clustering, irrespective of how it

[2] Masouredis, S. P., E. J. Sudora, L. Mahan, and E. J. Victoria. Manuscript in preparation.

FIGURE 3 Ferritin distribution patterns on Rh-positive (R_1R_1) red cells. *a*. Incubated with [^{125}I]IgG anti-Rh$_o$(D), A-133 (0.20 μg N). Unagglutinated. *b*. Incubated with commercial, saline-active, complete IgM anti-Rh$_o$(D). Macroscopic agglutination scored at 1+. *c*. Incubated with [^{125}I]IgG anti-Rh$_o$(D), A-133 (0.20 μg N), and then treated with GA before staining with ferritin-conjugate. Unagglutinated. *d*. Incubated with [^{125}I]IgG anti-Rh$_o$(D), A-133 (0.20 μg N), plus commercial, incomplete IgG anti-Rh$_o$(D) and BSA. Macroscopic agglutination scored 3+. Final magnification 49,000 ×; bar signifies 0.5 μm.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is induced, there is a progressive saturation of IgG antigenic determinants by the anti-IgG with ratios greater than 5–6 as compared to 2–4 found with monodispersed cell-bound IgG.

With due allowance for the antigen clustering induced by conjugate staining, Rh hemagglutination was accompanied by redistribution of $Rh_o(D)$ determinants into large discrete aggregates. At this time, it is not possible to assess whether hemagglutination-associated clustering represents aggregation present before conjugate staining or if it reflects conjugate clustering of antigen sites due to enhanced mobility of membrane components in the ghost preparation. The latter possibility cannot be the complete explanation, since a recent study that employed direct staining by ferritin-conjugated anti-$Rh_o(D)$ also indicates that clustering is associated with agglutination (52). In that study, antigen clustering was observed by thin-section electron microscopy on agglutinated neuraminidase- and papain-treated red cells. As in this study, it was not apparent with incomplete IgG anti-$Rh_o(D)$ in albumin media. Voak, Cawley, Emmines, and Barker (52) have interpreted the results of enzyme-induced antigen clustering as due to limited membrane mobility caused by the enzyme treatment. In fact, our data, obtained with cross-linking reagents, suggest that antigen mobility is a fundamental property of the red cell membrane, operant whether clustering occurs or not. Thus, conditions that result in antigen clustering should not be viewed as "triggering off" (52) antigen mobility, but rather allowing the aggregation of normally mobile components of the membrane.

The role of albumin in hemagglutination is not fully understood and undoubtedly is complex. The conventional view is that it alters the dielectric constant, which results in a reduction of the zeta potential of the red cells (38). Recent evidence, however, has questioned this interpretation and has challenged the role of zeta potential as a major determinant force in hemagglutination (49). An alternative explanation is that albumin, like polymers such as neutral dextrans and polyamino acids, may act by serving as loosely bound cross-bridges between cells (21, 53).

On the basis of the ultrastructural evidence presented in this paper, there appear to be two different mechanisms involved in antibody-induced hemagglutination. One, exemplified by cells agglutinated with IgM antibodies, protease modification of the red cell, and the antiglobulin reaction, involves the clustering of antigen receptors. The other mechanism, typified by incomplete antibodies in the presence of albumin and probably other types of neutral polymers, results in hemagglutination that is not associated with marked clustering of antigen sites.

A simplistic explanation for hemagglutination associated with clustering is that the antibody cross-links two different cells by binding to a receptor on each cell monovalently. Clustering of antigen sites into patches results in a stronger cross-link, ostensibly due to weak interactions between adjacent antibody molecules. This process is facilitated in the enzyme-treated cells, ostensibly because of the increased freedom of mobility of $Rh_o(D)$ antigen sites in the plane of the membrane. In IgM-induced hemagglutination, the IgM molecule itself, being multivalent, traps migrating antigen sites within its immediate radius, initiating the production of a cluster.

In the antiglobulin-induced clustering, the bivalent anti-IgG would serve to anchor two migrating IgG-antigen complexes. There would follow a rapid multiplication and buildup of clusters, since each cell-bound IgG molecule has seven to eight antigenic determinants for anti-IgG. Because of this potential for rapid buildup of clusters, the antiglobulin reaction is probably a very efficient mechanism for producing antigen clusters and agglutination. Prevous work (7, 17) has, in fact, shown that the antiglobulin reaction is capable of agglutinating cells that contain only 100–500 molecules of cell-bound IgG.

Only a few ultrastructural studies have been concerned with the role of membrane fluidity in agglutination. While immunofluorescence techniques have been useful, only the resolution provided by electron microscopy is capable of revealing the dynamic behavior of discrete membrane markers. The paucity of electron microscopic evidence is, in part, due to technical difficulties in assessing antigen mobility in the plane of the membrane. Investigations of this type must be concerned with two-dimensional aspects of membrane structure. While it is possible to reconstruct the surface patterns of membrane markers from thin sections, it is a tedious process and has not been used extensively. The problem can be approached more directly by the technique employed in this investigation or by freeze-fracture electron microscopy. Guérin, et al. (12) have studied two cell lines of plasmocytoma cells and have shown that only the cell line agglutinable by con A displays a clustered distribution pattern of intramembranous par-

FIGURE 4 Red cell ferritin distribution patterns. *a*. Rh-positive (R_1R_1) red cell incubated with [^{125}I]IgG anti-$Rh_o(D)$, A-132 (0.31 μg N). Unagglutinated. *b*. Papain-modified, Rh-positive (R_1R_1) cell incubated with[^{125}I]IgG anti-$Rh_o(D)$, A-132 (0.31 μg N). Macroscopic agglutination scored 2+. *c*. Control Rh-positive (R_1R_1) cell incubated with BSA. Unagglutinated. *d*. Control Rh-negative (*rr*) cell incubated with [^{125}I]IgG anti-$Rh_o(D)$, A-132 (0.31 μg N). Final magnification 49,000 ×; bar signifies 0.5 μm.

ticles. Nicolson has shown that con A-mediated agglutination of trypsinized 3T3 fibroblasts also is associated with aggregation of con A sites (33).

Although the mobility and aggregation of ABO sites induced by agglutination has not been directly demonstrated, the movement of intramembranous particles that bear those antigens has been conclusively shown (37). Part of the difficulty in studying the influence of antigen mobility in ABO agglutination is the large number of antigen receptors in the ABO system. While estimates vary (8, 36), there are about one million A antigen sites per red cell. Clustering may be less apparent in the face of this heavy ABO site density than would be the case with the relatively sparse density in the Rh system.

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