

Ultrastructural Mapping of Methyldopa and Anti-D IgG Erythrocyte Antigen Receptors

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ABSTRACT The ultrastructural distribution pattern and site density of α -methyldopa immunoglobulin G (α -MD IgG) on the red cell membrane was observed and compared with that of anti-D IgG, with ferritin-conjugated rabbit anti-human IgG and [125 I]anti-D. α -MD IgG binds to all common types of human red cells, both Rho(D) positive and negative, to give a random, aperiodic distribution pattern grossly indistinguishable from the red cell D receptor site pattern. α -MD IgG inhibits the binding of [125 I]anti-D to D-positive red cells when the reaction is controlled with respect to total reaction volume, ionic strength, and the appropriate concentrations of the two IgG reactants. To determine if a α -MD IgG binds to the D-antigen receptor, D-positive red cells were sensitized with α -MD and [125 I]anti-D IgG separately and with both IgG preparations. The cell-bound radioactivity served to identify what proportion of the total ferritin-labeled IgG sites were due to anti-D. With nonsaturating concentrations of anti-D the number of IgG sites observed was equal to the sum of the sites found when the red cell was sensitized separately with α -MD and anti-D IgG. With saturating concentrations of anti-D there was a reduction in the expected number of IgG sites, indicating that α -MD IgG was excluded from binding. There was no comparable interaction of α -MD IgG and anti-D IgG when D-negative red cells were used. The results obtained indicate that α -MD IgG does not bind to the D antigen. The interaction between α -MD IgG and anti-D IgG for binding sites on the red cell membrane may be due to the close physical proximity of the two receptors, so as to produce steric hindrance in binding of the two IgG preparations when both are present. The α -MD IgG receptor appears to be a part of the Rh antigen complex that occurs in both D-positive and D-negative red cells and probably contains

receptors for other types of warm-antibody immune hemolytic anemias.

INTRODUCTION

About 15% of hypertensive patients treated with the antihypersensitive drug, methyldopa (α -methyl-3,4-dihydroxy-L-phenylalanine [Aldomet, Merck Sharp & Dohme, Division of Merck & Co., West Point, Pa.]), hereafter designated α -MD,¹ develop a positive direct antiglobulin reaction (Coombs), due to cell-bound IgG (1-4). In spite of the presence of a strongly positive direct antiglobulin reaction, less than 1% of the patients on α -MD therapy develop overt hemolysis (3, 4). In contrast to other types of drug-induced immune hemolysis, in which the drug participates in the immune reaction such as with penicillin (5, 6), α -MD does not participate in the interaction of the α -MD-induced IgG with red cells. As a result there is no serological test that can be used to establish definitively the diagnosis of α -MD-induced hemolysis. The diagnosis is based on the association of α -MD therapy, serological findings consistent with those found in warm antibody type autoimmune hemolytic anemia (AIHA), and the resolution of serological findings and hemolysis, if present, after cessation of drug administration.

In those patients with hemolysis both the clinical and serological picture is indistinguishable from the findings observed in "idiopathic" warm-antibody type AIHA. Serologically, α -MD IgG-containing eluates react optimally at 37°C with all common types of human red cells, both Rh positive and negative. They react poorly or not at all with Rh-null red cells (7, 8) and under

¹ Abbreviations used in this paper: AIHA, autoimmune hemolytic anemia; BNS, buffered normal saline, 1 part 0.15 M phosphate buffer to 9 parts normal saline; BSA, bovine serum albumin; EM, electron microscope; Fer-HGG, ferritin-conjugated rabbit anti-human IgG; α -MD, α -methyl-3,4-dihydroxy-L-phenylalanine.

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appropriate conditions appear to possess specificities for Rh antigens such as e and c (1, 8) suggesting that the α -MD induced red cell-binding IgG, like the IgG found in AIHA, is directed against the Rh antigen complex of the erythrocyte.

Studies reported previously showed that α -MD IgG under certain conditions, is capable of inhibiting the binding of [125 I]anti-D to D-positive red cells (9). In this paper the number and receptor site pattern of anti-D IgG and α -MD IgG-binding sites on the red cell membrane at the ultrastructural level was visualized with immunoferritin conjugates. These data were compared with the results obtained when both types of red cell-binding IgG preparations were used to sensitize the red cell to determine if the binding is to the same red cell receptor. [125 I]anti-D was used to provide an independent method of determining what proportion of the total cell-bound IgG molecules was due to anti-D IgG. A preliminary report has been presented in abstract form (10).

METHODS

Patient source of α -MD red cell-binding IgG. IgG containing eluates were prepared from the red cells of six patients who ranged in age from 27 to 81 yr and who had been treated for hypertension with α -MD from 1 to 5 yr. Four of the six patients were blood donors found to have a positive direct antiglobulin reaction due to IgG when their blood was processed. They had met the criteria for blood donation, but had neglected to disclose that they had been on α -MD therapy for hypertension, which was subsequently ascertained by a followup investigation. Two of the six patients had been hospitalized for a Coombs-positive, IgG-specific, hemolytic anemia (Hb 8.2 and 7.4 g/100 ml), and reticulocytosis. A presumptive diagnosis of α -MD-induced hemolytic anemia was made since there was no other clinical explanation for the hemolysis and there was clinical and serological resolution of the anemia within 3-6 mo after cessation of the α -MD administration. The patient group consisted of four women and two men; five were blood group 0, Rh positive, and one was blood group A, Rh negative.

Preparation of α -MD IgG eluates. The α -MD IgG containing eluates were obtained by acid elution of the patient's IgG-coated red cells by the method employed for the preparation of [125 I]anti-D IgG (9, 11). The eluates were concentrated about 10-fold by pressure dialysis and then equilibrated by dialysis against buffered normal saline (BNS), pH 6.5, containing 0.25% phenol and 1:10,000 merthiolate. The concentrated eluates were hemoglobin tinged and from 1 to 8 ml of packed red cells was used to prepare 1 ml of eluate. In some patients the red cell-binding α -MD IgG free in the plasma was isolated by sensitizing at 37°C for 1 h normal, washed, 0, Rh-positive red cells with the patient's plasma. The plasma α -MD IgG was then recovered by acid elution of the sensitized red cells as described above.

Characterization of the α -MD IgG eluates. The α -MD-containing eluates sensitized both Rh-positive and negative red cells to give 4 plus antiglobulin reactions. The antiglobulin titers ranged from 32 to 256 with an R₁R₂ cell and a commercial multivalent antiglobulin reagent. Eluates derived from the plasma α -MD IgG gave weaker reactions,

from 2 to 3 plus. There were weak (\pm to $+$) reactions, except for a 3 plus reaction with one eluate (Ber.) with both type A₂ and 0 frozen and fresh Rh-null red cells. The reaction with Rh-null cells in all cases was significantly less than that observed with random Rh-positive and negative human red cells. One eluate (Hil.) sensitized an LW-negative human red cell to give a 4+ antiglobulin reaction. The eluates reacted with both gorilla and chimpanzee erythrocytes to give 2-4 plus antiglobulin reactions. Immunoelectrophoresis of the highly concentrated eluates (50-100-fold) contained only IgG except for a faint trace of a protein in the α -globulin region. No IgM or IgA could be demonstrated. The IgG in the eluates contained both kappa and lambda light chains, as determined by double immunodiffusion. The IgG content of the highly concentrated eluates, as measured by quantitative radial immunodiffusion, was estimated to be in the range of 0.3-2.0 μ g N/ml.

Preparation of ferritin-conjugated antibodies. Ferritin-conjugated rabbit anti-human IgG (Fer-HGG) was prepared as described previously, with toluene-2,4-diisocyanate as a coupling agent (12, 13). The ferritin was repeatedly recrystallized with 5% CdSO₄. The rabbit anti-human IgG serum was adsorbed with washed human type 0, A, and B red cells and red cell stroma, after which the IgG was isolated by (NH₄)₂SO₄ precipitation and DEAE chromatography. The ferritin conjugate was separated from both unconjugated ferritin and IgG by column chromatography with Bio-Rad Agarose A-5m, 6% gel (200-400 mesh) (Bio-Rad Laboratories, Richmond, Cal.) equilibrated with 0.05 M phosphate buffer, pH 7.5. Appropriate fractions were pooled and the fraction containing the immunoferritin conjugate was identified with immunoelectrophoresis with goat anti-rabbit IgG and rabbit antiferritin. The conjugate was concentrated by either pressure dialysis or ultracentrifugation so as to contain 2-5 mg protein/ml before use. The conjugates, at this concentration, had titers that ranged from 16 to 64 when tested against R₁r red cells sensitized with a 1:5 dilution of a high titered anti-D serum.

Red cell-binding studies with [125 I]anti-D IgG and α -MD IgG. [125 I]anti-D was prepared as described previously (11). Washed red cells of different Rh phenotypes were reacted with various quantities of [125 I]anti-D IgG, α -MD IgG containing eluates, or with combinations of both types of red cell-binding IgG preparations. In a typical experiment, an aliquot of a 5-10% red cell suspension was incubated with the IgG preparation at 37°C for 1 h. The sensitized red cells were washed four times with an excess of cold BNS, pH 6.5 containing 0.3% bovine serum albumin (BSA), and the quantity of cell-bound 125 I was determined by well-type crystal scintillation counting. Results are expressed in micrograms. IgG anti-D nitrogen bound to 10¹⁰ red blood cells, calculated from the specific activity of the [125 I]anti-D IgG fraction, and by direct counting of the number of red cells in the cell suspension by a Coulter Counter, model F (Coulter Electronics Inc., Hialeah, Fla.). The cell-bound anti-D IgG nitrogen was converted to average number of IgG molecules per red cell, with a molecular weight of 171,000 for the IgG and a value of 13.13% for the nitrogen content of human IgG (14).

Immunoferritin conjugate staining of IgG-containing red cell membranes for electron microscopy. Red cells sensitized with either [125 I]anti-D IgG or α -MD IgG were lysed at an air-water interface and picked up from above on carbon-strengthened collodion-coated electron microscopy (EM) grids by lowering the grids onto the water surface as described by Nicolson, Masouredis, and Singer (13). The grids were conditioned with a solution of 5% BSA and

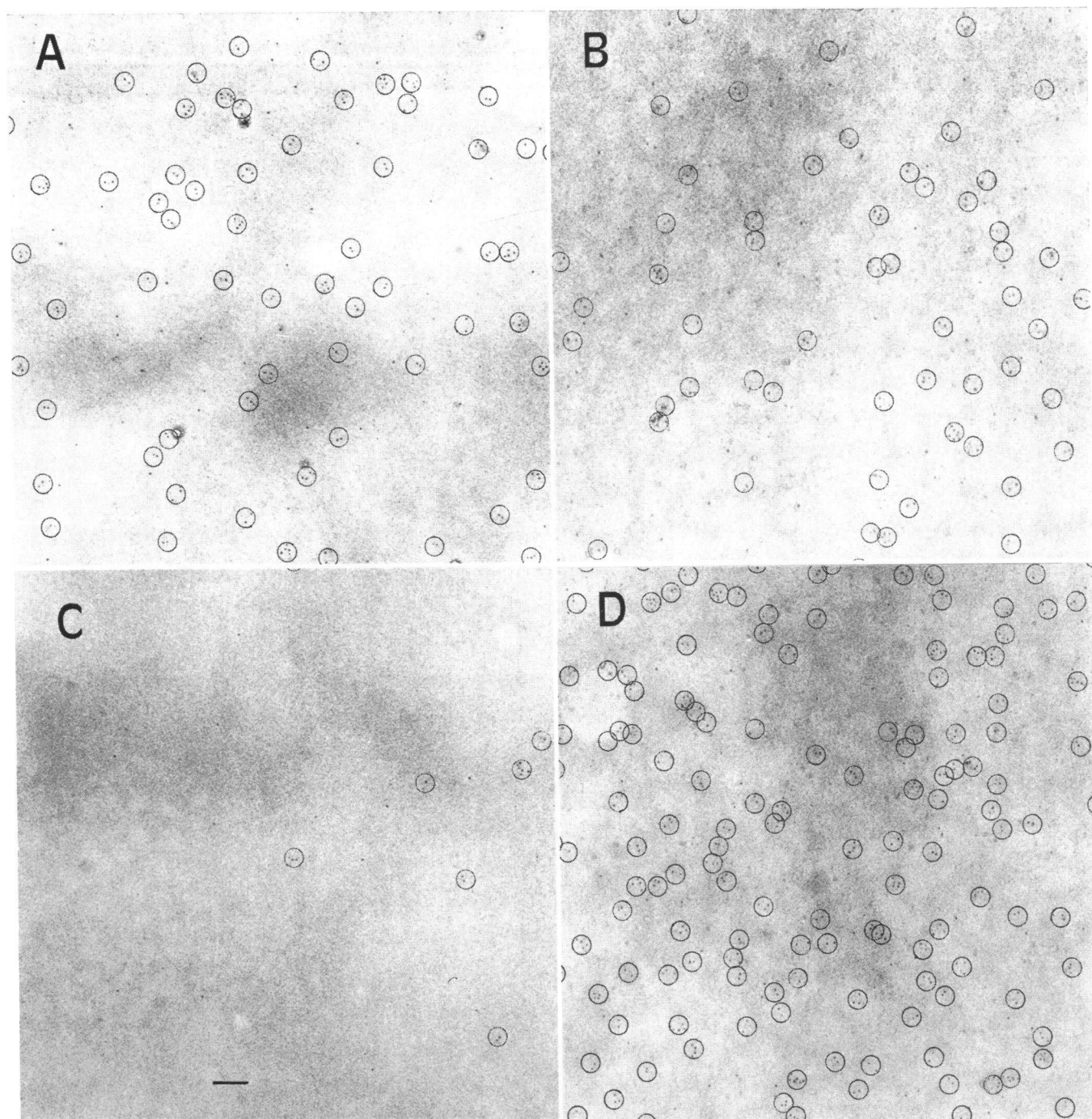


FIGURE 1 Electron micrographs of IgG-sensitized human Rh-positive (R_1R_1) red cells stained with Fer-HGG. A, red cell sensitized with [125 I]anti-D IgG. B, red cell sensitized with α -MD IgG (Jon.). C, control red cell. D, red cell sensitized simultaneously with both [125 I]-anti-D and α -MD IgG. Marker bar is 0.1 μ m. Clusters of ferritin are enclosed by circles of 300 Å radius.

stained by adding a drop of the immunoferritin conjugate to the EM grid. After 3–8 min of incubation at room temperature, the grids were washed by floating them face down on 6–10 fresh buffer surfaces, and finally distilled water.

The specimens were air-dried and examined in a Zeiss model 9S electron microscope (Carl Zeiss, Inc., New York).

Scoring of the electron micrographs was carried out by visually identifying clusters of ferritin containing two or

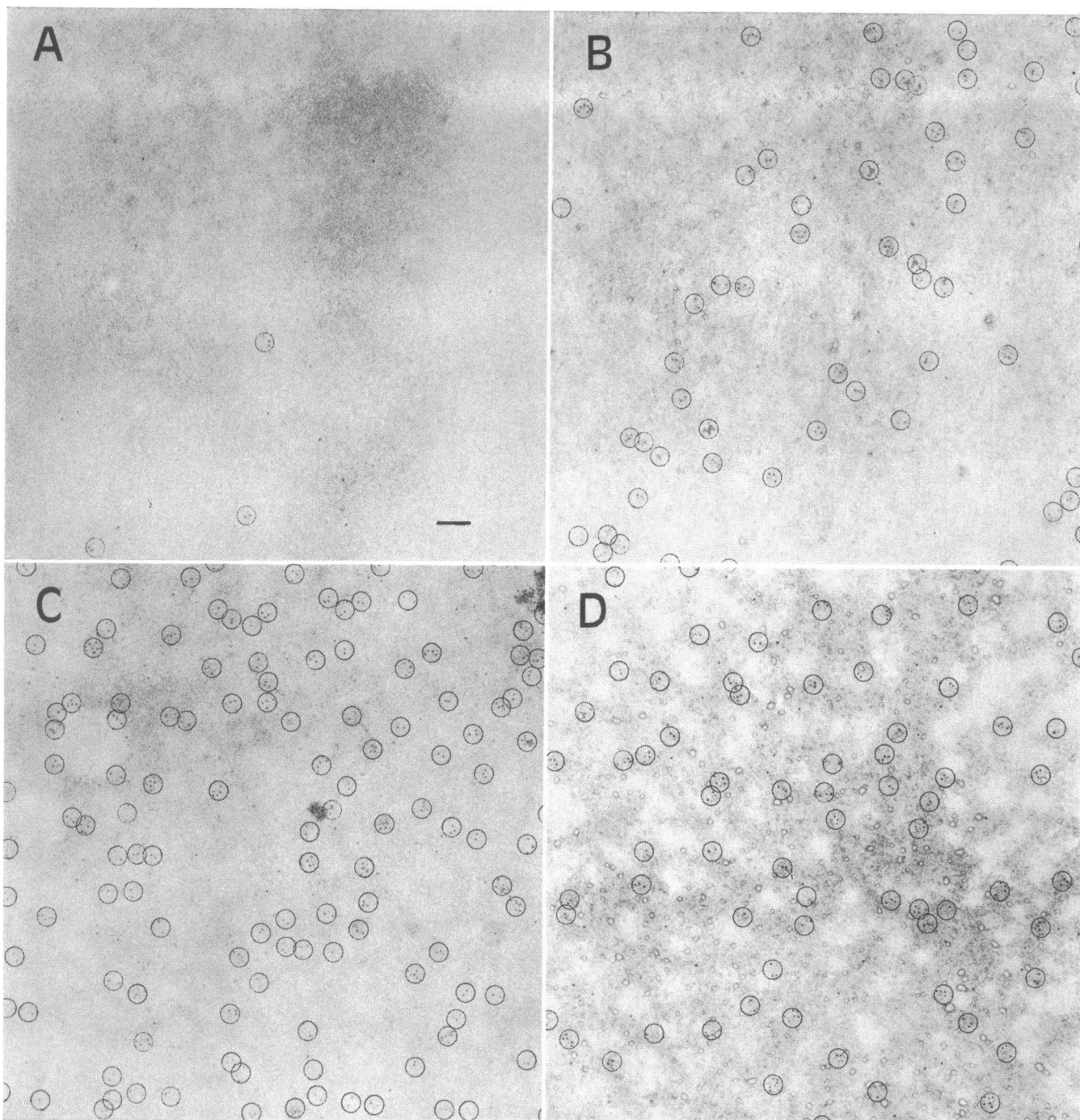


FIGURE 2 Electron micrographs of IgG-sensitized human red cells stained with Fer-HGG. A, Rh-negative (rr) red cell incubated with [125 I]anti-D IgG. B, Rh-negative (rr) red cell sensitized with α -MD IgG. C, Rh-positive (R_1R_1) red cell sensitized simultaneously with both [125 I]anti-D and α -MD IgG. D, Rh-negative (rr) red cell sensitized simultaneously with both [125 I]anti-D and α -MD IgG. Marker bar is 0.1 μ m. Clusters of ferritin are enclosed by circles of 300 Å diameter.

more ferritin particles and drawing 600-Å diameter circles around each such ferritin cluster, on the assumption that each human IgG molecule has several antigenic determinants

for rabbit anti-human IgG (15, 16). The number of ferritin particles per cluster for one experiment, in which 2,442 clusters were counted, was 52–71% had two; 18–36% had

TABLE I
α-MD and Anti-D IgG Red Cell Binding Sites

Red cell sensitization	Probable Rh genotype	Ferritin clusters counted	Membrane surface area counted	Ferritin clusters per μm^2 membrane	No. receptor sites per cell	
					Ferritin clusters per cell*	I* anti-D sites per cell
			μm^2			
α -MD IgG‡	R ₁ R ₁	796	25	31.8	4,620±300¶	—
α -MD IgG	rr	614	28	21.9	3,180±160	—
Control	R ₁ R ₁	647	23	3.6	520±120	—
Combined α -MD and anti-D IgG binding at relatively low anti-D saturation						
α -MD IgG§	R ₁ R ₁	1,836	75	24.5	3,550±110	—
α -MD IgG	rr	1,227	52	23.6	3,420±150	—
Control	R ₁ R ₁	197	37	5.3	770±80	—
Anti-D IgG	R ₁ R ₁	972	35	27.8	4,030±200	3,700±135
Anti-D IgG	rr	168	44	3.8	550±50	40±2
α -MD + anti-D	R ₁ R ₁	2,616	62	42.2	6,120±160	3,330±121
α -MD + anti-D	rr	1,362	57	23.9	3,460±120	30±2
Combined α -MD and anti-D IgG binding at relatively high anti-D saturation						
α -MD IgG	R ₁ R ₁	1,025	22	46.6	6,760±140	—
α -MD IgG	rr	1,382	44	31.4	4,550±170	—
Control	R ₁ R ₁	134	10	13.4	1,940±170	—
Anti-D IgG	R ₁ R ₁	995	23	43.3	6,270±200	7,940±284
Anti-D IgG	rr	61	16	3.8	550±84	9±0.5
α -MD + anti-D	R ₁ R ₁	646	15	43.1	6,240±170	6,830±241

* Based on cell surface area of 145 μm^2 (13).

‡ Han. α -MD eluate, conj. Z-1, I-125 anti-D preparation A-129.

§ Jon. α -MD eluate, conj. W-1, I-125 anti-D preparation A-128, electron micrographs shown in Figs. 1 and 2.

|| Jon. α -MD eluate, Conj. I-1, I-125 anti-D A-126.

¶ Mean ± SEM.

three; 4–12% had four; 2–8% had five; and 0.5–2.6% had six or more. At times ferritin aggregates were observed and in such cases the aggregates were not scored. The electron micrographs were routinely taken at 20,000 magnification and 20 or more micrographs were prepared for each cell study. Usually four to five micrographs of four to five different red cells were taken. The ferritin counting data, as presented in the accompanying tables, indicate the total number of ferritin clusters counted for each experiment. The value of 145 μm^2 was used for the total red cell surface area (13) in calculating the number of cell-bound IgG molecules per red cell.

RESULTS

Number and distribution pattern of α -MD IgG binding sites on erythrocyte membranes. Electron micrographs of α -MD IgG-sensitized red cells stained with Fer-HGG are shown in Figs. 1 and 2 (B). Both D-positive (R₁R₁) and D-negative (rr) red cells contain ferritin clusters that identify the presence of cell-bound IgG. The distribution of α -MD IgG binding sites on the membranes of both D-positive and negative red cells is randomly dispersed without any apparent periodicity, although there are localized areas with higher

concentrations of ferritin clusters, suggesting clumping of the sites. With the reaction conditions used in these experiments (cell concentration, α -MD IgG concentration, reaction volume, etc.), the number of α -MD IgG receptors per red cell ranged from 3,550 to 6,760 for three different R₁R₁ cells and from 3,180 to 4,550 for three different rr cells (Table I). Although the D-negative cell (rr) consistently bound less α -MD IgG (from 4 to 40% less), the difference was significant at the $P < 0.01$ level in only two of the three experiments. The distribution pattern of α -MD IgG binding sites on the red cell membrane is similar to the pattern of anti-D IgG binding to D receptor sites (Fig. 1A). The number of α -MD IgG binding sites on R₁R₁ red cell in this experiment was about the same as the number of D receptor sites observed when a nonsaturating concentration of anti-D was used to sensitize the R₁R₁ red cell (antigen excess).

Relationship of α -MD IgG binding sites to the D-receptor site. In view of the apparent similarity between the ultrastructural binding pattern of α -MD IgG and anti-D IgG, experiments were carried out to ascer-

tain if the α -MD IgG binds specifically to the D-receptor site of the Rh antigen complex. A D-positive cell was sensitized with [125 I]anti-D IgG and α -MD IgG separately and then with both types of IgG present simultaneously. If separate receptors exist for the α -MD and anti-D IgG, sensitization with both types of IgG should result in an increase in number of cell-bound IgGs, which would be equal to the sum of the IgG receptors found by sensitizing the red cell with each type of IgG individually.

Fig. 1 displays electron micrographs of an R₁R₁ red cell: sensitized with α -MD IgG (Fig. 1B); with non-saturating amounts of [125 I]anti-D IgG (Fig. 1A); and simultaneously with both types of IgG (Fig. 1D). An increase in the number of ferritin clusters is evident when the R₁R₁ red cell is treated simultaneously with α -MD and [125 I]anti-D IgG. The ferritin cluster counts for this experiment are presented in Table I. There are $3,550 \pm 110$ (mean \pm SEM) α -MD IgG ferritin clusters per cell, $4,030 \pm 200$ anti-D IgG ferritin clusters, and $6,120 \pm 160$ ferritin clusters when the R₁R₁ red cell is incubated with both anti-D and α -MD IgG. Of the 6,120 ferritin clusters per cell in the combined sensitization, 3,330 ferritin clusters could be attributed to anti-D binding by using the cell-bound radioactivity. Within experimental error and under nonsaturating quantities of anti-D, the observed number of cell-bound IgG molecules on the electron micrographs is equal to the sum of the IgG receptors found by sensitizing the red cell with α -MD IgG and anti-D IgG separately.

These results do not either establish or exclude the possibility that the D antigen is the specific receptor for α -MD IgG. Additive recovery could be due to α -MD IgG and anti-D IgG binding to different red cell receptors. An equally tenable interpretation is that α -MD IgG binds to the D receptor. Additive recovery would be expected in the presence of nonsaturating anti-D concentrations, since there would be insufficient anti-D present to block all the D receptors. That there is competition or interaction between α -MD IgG and anti-D IgG for a common receptor is indicated by the 11% reduction in anti-D IgG binding in the presence of α -MD IgG.

The results obtained with combined α -MD IgG and anti-D IgG binding with high concentration of anti-D are shown in Table I. The number of ferritin clusters observed after sensitizing the cell with both types of IgG is less than if both anti-D IgG and α -MD IgG bind to independent sites. There were $6,760 \pm 140$ IgG sites per cell when the red cell was treated with α -MD IgG alone, $6,270 \pm 200$ D sites when the red cell was treated with anti-D IgG alone, and $6,240 \pm 170$ IgG sites when the red cell was sensitized simultaneously with both anti-D and α -MD IgG. The number of ferritin clusters

in these three experiments did not differ significantly from each other. From the cell bound radioactivity $6,830 \pm 241$ of the 6,240 ferritin-marked IgG sites, or essentially all, were due to anti-D IgG, indicating that at high anti-D concentrations α -MD IgG is excluded from binding. These data and other experiments in which similar results were obtained with α -MD IgG eluates obtained from different patients indicate an interaction or competition between α -MD IgG and anti-D IgG for the same D red cell receptor.

The control experiments for these studies consisted of the same R₁R₁ red cell incubated in buffer and a D-negative red cell sensitized separately with each type of IgG and with both simultaneously. Background ferritin clusters on the buffer-incubated R₁R₁ red cell, presumably due to nonspecific binding of ferritin anti-human IgG, ranged from 520 to 1,940 clusters/cell (Table I). D-negative red cells incubated with [125 I]-anti-D IgG stained with the immunoferritin conjugate contained about 550 ferritin clusters/cell, of which less than 50 were due to cell-bound anti-D IgG, as judged from the cell-bound radioactivity. As expected, anti-D IgG does not bind to D-negative red cells, as evidenced by the lack of cell-bound radioactivity and the absence of significant ferritin clusters in the electron micrographs. In marked contrast, α -MD IgG shows significant binding to Rh-negative red cells.

D-negative red cells treated separately with anti-D IgG and α -MD IgG and with both types of IgG simultaneously were used as controls for the combined sensitization studies of D-positive red cells. There were $3,460 \pm 120$ ferritin clusters/cell when a D-negative red cell was sensitized with α -MD IgG in the presence of anti-D IgG, as compared to the 3,420 ferritin clusters/cell observed when α -MD IgG alone was used to sensitize the Rh-negative red cell (Table I). There is no binding interaction between α -MD IgG and anti-D IgG on a D-negative red cell, such as was observed when a D-positive red cell was sensitized with both types of IgG.

The expected and observed values for the combined studies are summarized in Table II.

Fig. 3 presents the frequency distribution of the number of ferritin clusters per square micrometer area of the red cell membrane for the study at relatively low concentration of anti-D IgG. Areas from three to six different red cells were scored for ferritin clusters. There is considerable variation in number of ferritin clusters per square micrometer surface area, particularly evident in the R₁R₁ anti-D-treated cell and in the R₁R₁ cell treated with both anti-D and α -MD IgG. The additive recovery of IgG sites on the R₁R₁ cell treated with both IgG preparations and the absence of a sig-

TABLE II
Interaction between Anti-D and α -MD IgG for
Binding Sites on the Red Cell

IgG used for sensitization	Number of ferritin clusters (IgG molecules) per cell		rr
	R ₁ R ₁	R ₂ R ₂	
Anti-D	4,030 \pm 200§	6,270 \pm 200	550 \pm 50
α -MD	3,550 \pm 110	6,760 \pm 140	3,420 \pm 150
No. expected (anti-D + α -MD)	7,580	13,030	3,970
No. observed (anti-D + α -MD)	6,120 \pm 160	6,240 \pm 170	3,460 \pm 120
I* anti-D sites	3,300 \pm 121	6,830 \pm 340	30 \pm 2
Calculated α -MD sites	2,820	0	3,430

* Jon. α -MD eluate, conj. W-1, I-125 anti-D preparation A-128.

† Jon. α -MD eluate, conj. L-1, I-125 anti-D preparation A-126.

§ Mean and SEM.

nificant increase over the α -MD IgG binding on a similarly treated rr cell is evident.

Inhibition of anti-D IgG red cell binding by α -MD IgG. Experiments were carried out to define the conditions affecting the inhibition of [¹²⁵I]anti-D binding to red cells by α -MD IgG to facilitate the interpretation of the ultrastructural studies based on the immunoferritin conjugate.

In the experiments presented in Table I, α -MD IgG reduces the binding of [¹²⁵I]anti-D by some 10–15%. Table III shows the concentration dependence of α -MD IgG inhibition of [¹²⁵I]anti-D binding to D-positive red cells. The results shown were obtained with three different D-positive red cells of different Rh phenotype and the α -MD IgG eluates were obtained from three different donors. The reduction in binding of labeled anti-D appears to be proportional to the quantity of α -MD IgG present during the reaction. As the volume of α -MD IgG eluate is increased, there is progressive inhibition of anti-D binding, reaching levels of 32–50% reduction in cell-bound anti-D when the α -MD IgG eluate volume is increased to 0.7 ml. The anti-D concentration used was about 70% of the saturating concentration.

Table IV shows that α -MD IgG inhibition of anti-D binding is dependent on the ionic strength of the reaction. At low ionic strength, there is little or no inhibitory activity of the α -MD IgG eluates, whereas at an ionic strength of 0.15, the same α -MD IgG produces a 30–40% reduction in anti-D bindings.

Table V shows the inhibitory activity of three different α -MD IgG eluates on the binding of anti-D to D-positive red cells of six different Rh phenotypes. The red cells for each experiment were obtained from dif-

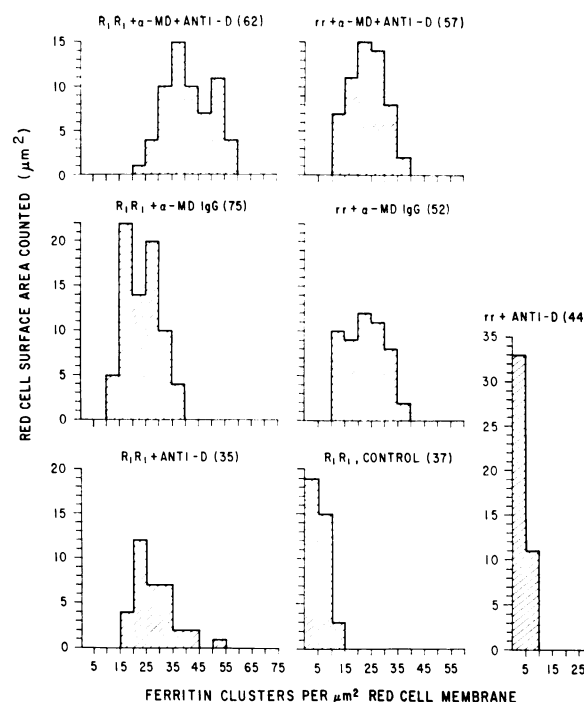


FIGURE 3 Frequency distribution of the number of ferritin clusters per square micrometer red cell surface area of IgG-sensitized human red cells stained with Fer-HGG. Caption in each frame indicates the type of red cell and IgG used for sensitizing. The number in parentheses is the total number of square micrometers surface area counted.

ferent, unrelated donors. No readily discernable pattern of α -MD IgG inhibition of anti-D binding which re-

TABLE III
Concentration of α -MD IgG and Inhibition of
Anti-D IgG Red Cell Binding

α -MD IgG added	Reduction in anti-D binding		
	R ₂ R ₂ * (ml)	R ₂ R ₂ * (%)	R ₁ R ₁ §
0.05	2	11	—
0.10	9	10	1
0.20	18	13	6
0.30	—	19	7
0.35	32	—	—
0.40	—	22	—
0.50	—	29	18
0.70	—	50	32

0.1 ml of a 5% red cell suspension was used in each experiment.

* Hil. α -MD eluate, I-125 anti-D (A-103), control R₂R₂ bound 5.43 μ g N/10¹⁰ red cells.

† Ber. α -MD eluate, I-125 anti-D (A-104), control R₂R₂ bound 2.68 μ g N/10¹⁰ red cells.

§ Rio. α -MD eluate, I-125 anti-D (A-105), control R₁R₁ bound 5.19 μ g N/10¹⁰ red cells.

TABLE IV
Effect of Ionic Strength on α -MD IgG Inhibition
of Anti-D Red Cell Binding

α -MD IgG	μ g N bound per 10^{10} red cells			Reduction in anti-D binding
	Ionic strength	Control red cells	With α -MD IgG	
				%
Hes. plasma	0.15	2.73	1.65	40
Hes. cells	0.15	2.73	1.72	37
Ber. cells	0.15	2.73	1.90	31
Hes. plasma	0.06	5.55	5.39	3
Hes. cells	0.06	5.55	5.32	4
Ber. cells	0.06	5.55	5.61	0

Plasma indicates that the α -MD IgG was obtained from the plasma with normal red cells to prepare the eluate, and cells indicate that the eluate was prepared from the patient's coated red cells. 0.3 ml of the α -MD IgG was used in each experiment and 0.1 ml of a 5% R₁R₂ red cell suspension. [¹²⁵I]anti-D preparation A-104. Ionic strength was reduced by the addition of 0.29 M glycine.

lates to the Rh antigen complex in these different Rh-positive red cells is evident. Although inhibition of anti-D binding in some experiments appears to correlate with the D antigen content of the target red cell, the highest (40%) was associated with a low D-antigen content red cell (R₀r) and in another experiment about the same degree of inhibition was found with both a

high (R₂R₂, 30%) and a low (R₀r, 29%) anti-D binding red cell. α -MD IgG inhibition of anti-D binding does not appear to be directly related to the D antigen content of the red cell.

There is a marked reduction in the inhibition of anti-D binding by α -MD IgG at high saturating concentrations of anti-D (Table VI). When 0.1 ml of [¹²⁵I]anti-D was used, α -MD IgG reduced the binding of anti-D for an R₁R₁ red cell by 41%, whereas when 1.5 ml of [¹²⁵I]anti-D was used there was only a 9% reduction in anti-D binding. In another experiment an R₁R₂ red cell was incubated with 0.4 ml of α -MD IgG and progressively increasing quantities of [¹²⁵I]anti-D. Inhibition of anti-D binding progressively decreased as the anti-D concentration was increased (from 14% at 0.1 ml anti-D to 4% with 1.0 ml anti-D).

DISCUSSION

The red cell-binding IgG associated with the administration of α -MD bears a striking resemblance to the warm-type of erythrocyte "autoantibody" found in patients with AIHA. A feature common to both disorders is that the red cell-binding IgG shows an Rh-related specificity. The α -MD IgG, in some patients, has a partial specificity for Rh antigens such as e and c; reacts poorly or not at all with Rh-null red cells, and binds to the red cells of lower primates that contain Rh antigens (3, 7, 8, 17-20). The serological and hematological changes associated with the administration of α -MD represents a potentially useful model for

TABLE V
Relationship between Rh Phenotype and Inhibition of [¹²⁵I]Anti-D
Red Cell Binding by α -MD IgG Eluates

Probable Rh phenotype	α -MD IgG eluate					
	Hil. cells*		Hes. cells†		Rio. cells§	
	N bound	Reduction	N bound	Reduction	N bound	Reduction
		%		%		%
R ₂ R ₂	3.01	30	—	—	—	—
R ₁ R ₂	2.87	23	2.00	14	6.99	26
R ₁ R ₁	1.99	24	2.47	40	5.31	27
R ₂ r	1.63	29	1.65	6	6.05	28
R ₁ r	—	—	1.10	9	3.28	25
R ₀ r	—	—	—	—	1.94	40

0.1 ml of a 5% red cell suspension was used in all tests. Red cells for each study were obtained from different unrelated donors.

* Preparation A-103, 0.34 μ g anti-D used/test and 0.3 ml α -MD IgG eluate, total reaction volume 1.2 ml.

† Preparation A-104, 0.30 μ g anti-D used/test and 0.3 ml α -MD IgG eluate, total reaction volume 1.2 ml.

§ Preparation A-105, 2.34 μ g anti-D used/test and 0.5 ml α -MD IgG eluate, total reaction volume 1.6 ml.

|| μ g N bound per 10^{10} red cells to control red cell suspension.

TABLE VI
Relationship between [125 I]Anti-D Concentration and Inhibition of
[125 I]Anti-D Red Cell Binding by α -MD IgG

Probable red cell phenotype	High anti-D concentration* N bound§			Low anti-D concentration‡ N bound§		
	Control	With α -MD	Percent reduction	Control	With α -MD	Percent reduction
R ₁ R ₁	1.16	0.681	41	2.45	2.23	9
rr	0.057	0.055	4	0.106	0.113	7¶

* 1.5 ml [125 I]anti-D, preparation A-129, 2.43 μ g anti-D used per test.

‡ 0.1 ml [125 I]anti-D, preparation A-129, 0.162 μ g anti-D used per test.

§ μ g N bound to 10^{10} red cells.

|| 0.3 ml α -MD IgG eluate, Hes. cells.

¶ The 7% increase in [125 I]anti-D binding on the Rh-negative red cell in the presence of α -MD IgG is not significant, since the counting rates were less than two times the background.

the study of AIHA that may provide insights into such questions as the role of the Rh antigen complex in "autoimmune" hemolytic states and the paradox of apparently normal in vivo survival of red cells heavily coated with IgG. Of interest is that L-3,4-dihydroxy-phenylalanine (L-dopa), a drug used for symptomatic control of Parkinsonism that is structurally related to α -MD, produces a similar serological picture (21). In view of these observations, the present studies were carried out to identify at the ultrastructural level the red cell α -MD IgG receptor sites and to determine if α -MD IgG binds specifically to the red cell D antigen, one of the major antigens in the Rh complex.

Immunoferritin conjugates and electron microscopy have been used to stain IgG-sensitized red cells to visualize the receptor site pattern and density of Rh antigens (22, 23), the A antigens (22, 24, 25), and autoantibodies (23). The methods used involved the thin sectioning of the conjugate-stained red cells so that the ferritin-marked IgG sites were visualized in only one plane, i.e., at the periphery of each red cell section. The methods used in this report differ from those used previously in that red cell membranes were visualized in two dimensions, and that [125 I]anti-D IgG provided an independent radioactive label useful both for confirming the ferritin counting data and for providing a means of measuring the quantity of anti-D IgG bound in the presence of α -MD IgG.

There are a number of limitations on the use of immunoferritin conjugates that should be considered before proceeding with the interpretation of the data. An obvious one is the tendency of conjugates to bind nonspecifically to red cell membranes. With good preparations there were about 500 nonspecific sites/cell, but in some studies nonspecific binding was considerably higher. There is a reduction in antibody activity after conjugation of ferritin to anti-IgG (26) which in these

studies was manifested by a 4- to 5-tube reduction in the antiglobulin titer. Another potential problem is the unconjugated anti-IgG in the chromatographed immunoferritin conjugate, which would lead to an underestimation of the cell-bound IgG. This source of error was controlled by comparing the ferritin sites with the number expected from the cell-bound radioactivity when [125 I]anti-D IgG was used alone (13). In this previous study the estimate of red cell D-antigen sites with immunoferritin conjugates was in good agreement with the number of D sites determined independently with [125 I]anti-D binding over a range of antigen site density from 10,000 to 20,000/red cell. The presence of IgG ferritin oligomers in the conjugate would also affect the interpretation of the data (25). With the conjugates used in these studies, however, less than 3% of the ferritin clusters contained six or more ferritin molecules.

The relatively large variation in antigen site density from cell to cell, which is graphically evident in Fig. 1 and which has been described by Voak and Williams (24), may be due to technical limitations of the method or may represent true red cell heterogeneity with respect to IgG binding. This variation in staining may be due to differences associated with in vivo red cell age heterogeneity or may in part be due to antigen mobility (13). Except for EM and autoradiograph of individual red cells, such cell-to-cell differences would not be detectable with standard serological techniques, in which the IgG bound per cell represents an average value calculated from the quantity of IgG bound to a large number of cells.

Single ferritin molecules were not scored as IgG binding sites even though they may represent cell-bound IgG in which the IgG-to-anti-IgG combining ratio is one. Justification for handling the data in this fashion is based on the agreement between cell-bound

radioactivity and ferritin cluster counts in which only clusters with two or more ferritin molecules were scored as one IgG site. This agreement may be fortuitous if there is a systematic underestimation of the cell-bound anti-D IgG as calculated from the cell-bound radioactivity, such as the assumption that the specific radioactivity of the anti-D IgG present in the eluate is the same as that of the total IgG fraction used in the radioiodination procedure. The number of single ferritin molecules varied from study to study, from very few in some to up to 10–15% of the clusters scored in others. It is conceivable that the decision to exclude single ferritin molecules has resulted in a 10–15% underestimation of the red cell IgG binding sites.

The possible distortion of antigen site distribution that may result from the osmotic hemolysis used to prepare the erythrocyte membranes for conjugate staining cannot be assessed with the techniques used in this study. Current models of membrane structure (27, 28) postulate continuous active lateral mobility of antigen containing proteins in the lipid bilayer, so that the position of the IgG binding sites is that fixed at the time the membranes were stained. Although there is no overt pattern to the distribution of IgG binding sites, it is evident (Figs. 1 and 2) that there is a random clumped or "contagious" distribution of sites, similar to that reported for A antigen sites on the red cell (24, 25). A variety of distribution patterns would be expected, since the pattern observed depends on the position of the actively moving components at the time the membrane was fixed.

This communication demonstrates unequivocally that at the ultrastructural level α -MD IgG binds to both Rho(D) positive and negative red cells with a site distribution pattern grossly indistinguishable from that observed with anti-D IgG on D-positive red cells.

α -MD IgG differs from anti-D, which binds only to D-positive red cells. The pattern is aperiodic and discontinuous, with regions that appear to have a higher density of sites than others. The number of α -MD IgG sites on D-negative red cells appear to be less than on the D-positive red cell, but the data are not statistically conclusive. The number of α -MD IgG sites per red cell ranged from 4 to 7,000, values similar to those found for D receptor sites on D-positive red cells with low D antigen content. These values probably do not represent saturation values and it is conceivable that with optimum reaction conditions, in which an excess of α -MD IgG is used, the number of α -MD IgG receptor sites may be considerably higher.

α -MD IgG, under certain conditions, inhibits the binding of [125 I]anti-D to D-positive red cells. Inhibitory activity appears to be directly proportional to the concentration of α -MD IgG (Table III), but unrelated

to the D-antigen content of the target red cell (Table V). The α -MD IgG inhibition of anti-D binding can be overcome by lowering the ionic strength of the reaction medium, by using saturating concentrations of [125 I]anti-D, or by increasing the reaction volume. As the reaction volume is increased from 1.2 to 4.2 ml by the addition of BNS, the inhibition of anti-D binding decreases from 37 to 8%. α -MD IgG is less effective in blocking anti-D binding to gorilla or chimpanzee red cells (9), even though both anti-D IgG (19, 20) and α -MD IgG bind to these nonhuman primate red cells (7). It is unlikely that the inhibition of anti-D binding by the α -MD IgG eluates is due to the presence of free Rh antigen in the eluate. One of the most potent α -MD IgG eluates used in these studies was obtained from an Rh-negative patient (Hil.) with α -MD-induced hemolytic anemia.

An explanation for the α -MD IgG inhibition of anti-D binding consistent with the experimental data obtained is that both IgGs bind to the same receptor, namely the D antigen. The ultrastructural distribution pattern is similar for both anti-D and α -MD IgG, and the reduction of α -MD IgG inhibitory activity produced by optimizing the reaction conditions for anti-D binding could be due to the relatively high average association constant (K_o) of the [125 I]anti-D (28).

The data based on the use of immunoferritin conjugates to stain IgG receptor sites on D-positive red cells sensitized simultaneously with both α -MD and anti-D IgG also are consistent with the interpretation that α -MD IgG binds to the D receptor. At nonsaturating concentrations of anti-D the number of ferritin-labeled IgG sites equals the sum of those found when the red cell is sensitized separately with α -MD and anti-D IgG, indicating that in antigen excess there are sufficient D receptors to accommodate both α -MD IgG and anti-D IgG. At saturating concentrations of anti-D the total number of ferritin-marked IgG sites does not exceed the number of anti-D sites, as determined from cell-bound radioactivity, indicating that under these conditions α -MD IgG is excluded from binding. The reduction in the number of IgG ferritin-labeled sites when both red cell-binding IgGs are used to sensitize the red cell is not due to inability of the anti-D IgG to bind specifically at high concentrations. Ferritin-labeled sites in excess of 35,000/cell have been observed with high D antigen content red cells (D⁺) sensitized with anti-D (unpublished observations).

In spite of the apparent D specificity of the α -MD IgG, the evidence against this interpretation is compelling. There are marked differences in the inhibition of [125 I]anti-D binding by α -MD IgG and unlabeled anti-D. At low [125 I]anti-D concentrations, unlabeled anti-D produces a significantly greater inhibition of

[¹²⁵I]anti-D binding than does α -MD IgG. Furthermore, at low ionic strength there is an increase in inhibitory activity of unlabeled anti-D, whereas the inhibition produced by α -MD IgG is reduced or completely abolished. The α -MD IgG inhibition of [¹²⁵I]anti-D binding to red cells of different Rh phenotype does not appear to be directly related to the D-antigen content of these cells (Table V). One α -MD IgG eluate that produced a 26–28% inhibition of anti-D binding to high D-antigen content red cells (R₁R₂, R₂r) effected a 40% reduction in anti-D binding to a low D-content red cell (R₀r). A different α -MD IgG eluate inhibited anti-D binding by 40% on a R₁R₁ red cell, but only produced a 6% reduction when an R₂r red cell was used. The serological specificity of α -MD IgG eluates is directed against the Rh antigen complex, but does not show any clearly defined specificity for the D antigen. Bakemeier and Leddy (8) found four separable serological antibody activities, two to what was postulated to be the Rh “nucleus” or precursor Rh substance, and one each against the e and c Rh antigens. They also showed that there are distinct serological differences in Rh-related antigenic specificities from one α -MD eluate to another. The results obtained with α -MD IgG inhibition of [¹²⁵I]anti-D binding to simian red cells lends additional support to the non-D-related specificity of α -MD IgG. There is little or no inhibition of anti-D binding to D-containing simian red cells by α -MD IgG (9). The most telling evidence that the D antigen is not the receptor for α -MD IgG is the demonstration at the ultrastructural level that α -MD IgG binds specifically to D-negative red cells. That this binding is immunologically significant is supported by the ability of α -MD IgG-sensitized D-negative red cells to agglutinate on the addition of anti-human IgG (Coombs).

An alternative explanation of the data that would accommodate the apparently contradictory observations is that α -MD IgG binds to a site different from the D receptor. Under this interpretation the α -MD IgG receptor is a part of the Rh-antigen complex, which is closely situated to the D-antigen receptor. The interaction between α -MD IgG and anti-D IgG would result from the close physical proximity of the two different receptors so that only one or the other IgG, but not both, could occupy their corresponding receptor site. Alternatively there may be a sterically imposed constraint on the ability of ferritin anti-IgG to bind to two or more closely situated cell-bound IgG molecules. The data on the α -MD IgG inhibition of [¹²⁵I]anti-D binding indicate that the interference in binding occurs at the level of IgG binding to the red cell, rather than with the binding of ferritin anti-IgG to IgG. It is unlikely that the IgG site densities of less than 10,000/cell prevailing in these experiments exceeded the resolution

of the immunoferritin method, since densities in excess of 25,000 have been observed with other Rh antibodies (unpublished observations). This method of molecular mapping has been used to study the interaction of [¹²⁵I]-anti-D with antibodies to the other Rh antigens. Preliminary results indicate a marked reduction in the expected recovery of IgG sites when antibodies to other Rh antigens are used simultaneously with [¹²⁵I]-anti-D to sensitize the target red cell (unpublished observations). The validity and potential utility of this technique as a general method for mapping red cell antigen receptors must await more detailed studies on molecular packing and independent determination of the molecular resolution of the method.

There is indirect evidence to indicate that the D antigenic determinant is protein (13). On the basis of results obtained with chemical labeling and treatment with proteolytic enzymes, only two of the major protein components resolved electrophoretically on sodium dodecyl sulfate polyacrylamide gels occur at the external surface of the red cell membrane (29). One is a sialoglycoprotein with a provisional molecular weight of about 50,000 which bears the ABH, Ii, and MN blood group receptors as well as receptors for phytohemagglutinin, wheat germ agglutinin, and influenza virus (30). In view of the marked differences between the ABH and Rh blood group systems, it is unlikely that this sialoglycoprotein carries the Rh antigens. Among the more notable differences are: (a) number of antigen sites per red cell, 10⁶ for A sites and less than 10⁵ for D sites (11, 16, 25); (b) tissue distribution; and (c) a significant difference with respect to the role they play in maintaining the structural integrity of the red cell, which has been discussed by Levine, Tripodi, Struck, Zmijewski, and Pollack (31). Absence of ABH substances, as in Bombay type blood, does not appear to be associated with hemolysis, whereas absence of Rh in Rh-null bloods leads to a decreased red cell survival.

The other major protein, designated component *a*, has a molecular weight of 105,000 and it is estimated that there are about 500,000 such molecules per cell (32). Component *a* is a likely candidate for the Rh antigens. The major difficulty is that there are 5–15 times more molecules of component *a* than D sites per red cell. The possibility exists, however, that the conformation of component *a* in the membrane is such that not all D sites would be available for anti-D binding at the cell surface. Alternatively, it is conceivable that the Rh antigens and the closely related α -MD IgG receptor occur on some other minor protein component of the red cell membrane. Definitive studies will be required to identify unequivocally the red cell protein that bears these antigens. It is tempting to speculate that the receptors for the other types of warm-

type autoantibodies will also be associated with the same Rh-bearing macromolecular component of the red cell membrane.

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REFERENCES

- Carstairs, K. C., S. M. Worlledge, C. T. Dollery, and A. Breckenridge. 1966. Methylidopa and hemolytic anemia. *Lancet*. 1: 201.
- Carstairs, K. C., A. Breckenridge, C. T. Dollery, and S. M. Worlledge. 1966. Incidence of a positive direct Coombs test in patients on α -methylidopa. *Lancet*. 2: 133-135.
- Worlledge, S. M., K. C. Carstairs, and J. V. Dacie. 1966. Autoimmune haemolytic anaemia associated with α -methylidopa therapy. *Lancet*. 2: 135-139.
- Worlledge, S. M. 1969. Annotation. Autoantibody formation associated with methylidopa (Aldomet) therapy. *Br. J. Haematol.* 16: 5-8.
- Petz, L. D., and H. H. Fudenberg. 1966. Coombs-positive hemolytic anemia caused by penicillin administration. *N. Engl. J. Med.* 274: 171-178.
- Swanson, M. A., D. Chanmougan, and R. S. Schwartz. 1966. Immunohemolytic anemia due to antipenicillin antibodies. Report of a case. *N. Engl. J. Med.* 274: 178-181.
- LoBuglio, A. F., and J. H. Jandl. 1967. The nature of the alpha-methylidopa red-cell antibody. *N. Engl. J. Med.* 276: 658-665.
- Bakemeier, R. F., and J. P. Leddy. 1968. Erythrocyte autoantibody associated with alpha-methylidopa: heterogeneity of structure and specificity. *Blood J. Hematol.* 32: 1-14.
- Masouredis, S. P., U. Marzec, E. Sudora, and M. K. Chalos. 1972. Methylidopa-induced erythrocyte binding IgG: another model of nonspecific attachment of immunoglobulin to erythrocytes. *Haematologia*. 6: 285-293.
- Masouredis, S. P. 1973. Quantity and Rh specificity of RBC binding sites for α -methylidopa IgG autoantibodies. *Blood J. Hematol.* 42: 1007. (Abstr.).
- Masouredis, S. P., M. E. Dupuy, and M. Elliot. 1967. Relationship between Rh₀(D) zygosity and red cell Rh₀(D) antigen content in family members. *J. Clin. Invest.* 46: 681-694.
- Singer, S. J., and A. F. Shick. 1961. The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. *J. Biophys. Biochem. Cytol.* 9: 519-537.
- Nicolson, G. L., S. P. Masouredis, and S. J. Singer. 1971. Quantitative two-dimensional ultrastructural distribution of Rh₀(D) antigenic sites on human erythrocyte membranes. *Proc. Natl. Acad. Sci. U. S. A.* 68: 1416-1420.
- Edelman, G. M., and W. E. Gall. 1969. The antibody problem. *Annu. Rev. Biochem.* 38: 415-466.
- Constantoulakis, M., N. Costea, R. S. Schwartz, and W. Dameshek. 1963. Quantitative studies on the effect of red-blood-cell sensitization on in vivo hemolysis. *J. Clin. Invest.* 42: 1790-1801.
- Rochna, E., and N. C. Hughes-Jones. 1965. The use of purified ¹²⁵I-labelled anti- γ globulin in the determination of the number of D antigen sites on red cells of different phenotypes. *Vox Sang.* 10: 675-686.
- Weiner, W., and G. H. Vos. 1963. Serology of acquired hemolytic anemias. *Blood J. Hematol.* 22: 606-613.
- Vos, G. H., L. D. Petz, G. Garratty, and H. H. Fudenberg. 1973. Autoantibodies in acquired hemolytic anemia with special reference to the LW system. *Blood J. Hematol.* 42: 445-453.
- Masouredis, S. P., M. E. Dupuy, and M. Elliott. 1967. Distribution of the human Rh₀(D) antigen in the red cells of non-human primates. *J. Immunol.* 98: 8-16.
- Masouredis, S. P. 1971. Quantitative studies of the Rh₀(D) antigenic determinants on gorilla erythrocytes. *Transfusion (Phila.)*. 11: 270-280.
- Henry, R. E., L. S. Goldberg, P. Sturgeon, and R. D. Ansel. 1971. Serologic abnormalities associated with L-dopa therapy. *Vox Sang.* 20: 306-316.
- Lee, R. E., and J. D. Feldman. 1964. Visualization of antigenic sites of human erythrocytes with ferritin-antibody conjugates. *J. Cell Biol.* 23: 396-401.
- Davis, W. C., S. D. Douglas, L. D. Petz, and H. H. Fudenberg. 1968. Ferritin-antibody localization of erythrocyte antigenic sites in immunohemolytic anemias. *J. Immunol.* 101: 621-637.
- Voak, D., and M. A. Williams. 1971. An explanation of the failure of the direct antiglobulin test to detect erythrocyte sensitization in ABO haemolytic disease of the newborn and observations on pinocytosis of IgG anti-A antibodies by infant (cord) red cells. *Br. J. Haematol.* 20: 9-23.
- Williams, M. A., and D. Voak. 1972. Studies with ferritin-labelled *Dolichos biflorus* lectin on the members and distribution of A sites on A₁ and A₂ erythrocytes, and on the nature of its specificity and enhancement with enzymes. *Br. J. Haematol.* 23: 427-441.
- Wernet, D., and A. Vogt. 1973. Antigen-binding capacity and affinity of ferritin conjugated antibodies. *Immunology*. 25: 687-695.
- Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. Cell membranes are viewed as two-dimensional solutions of oriented globular proteins and lipids. *Science (Wash. D. C.)*. 175: 720-731.
- Hughes-Jones, N. C. 1967. The estimation of the concentration and equilibrium constant of anti-D. *Immunology*. 12: 565-571.
- Bretscher, M. S. 1973. Membrane structure: some general principles. *Science (Wash. D. C.)*. 181: 622-629.
- Marchesi, V. T., R. L. Jackson, J. P. Segrest, and I. Kahane. 1973. Molecular features of the major glycoprotein of the human erythrocyte membrane. *Fed. Proc.* 32: 1833-1837.
- Levine, P., D. Tripodi, J. Struck, Jr., C. M. Zmijewski, and W. Pollack. 1973. Hemolytic anemia associated with Rhnull, but not with Bombay blood. *Vox Sang.* 24: 417-424.
- Bretscher, M. S. 1971. A major protein which spans the human erythrocyte membrane. *J. Mol. Biol.* 59: 351-357.