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Research Article

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Summary. The red cells of 63 members of 11 families were tested with ^{125}I–labeled anti-$Rh_0(D)$. Families with a history of hemolytic disease of the newborn due to fetomaternal Rh incompatibility were selected for study. In such families it was possible to determine the antibody binding to the $Rh_0(D)$ heterozygous red cells of the children and to compare within each family this value with the antibody bound to the father's $Rh_0(D)$–positive red cells and the mother's $Rh_0(D)$–negative red cells. The fathers in all the families studied could be assigned to two classes on the basis of the quantity of antibody bound to their red cells. One group bound about the same quantity of antibody to their cells as did their children, indicating that they were heterozygous for the $Rh_0(D)$ antigen. The other bound about twice as much antibody to their cells as did their children, indicating that they were homozygous for the antigen. The Rh genotype of the father in all 11 families could be ascertained by using the children in each family as a reference point. The members of two families showed a poor correspondence between antibody binding and zygosity. In one family an Rh heterozygous child (R^1r) took up 85% of the antibody bound to the father's homozygous cells (R^1R^1), and in the other family an Rh heterozygous child (R^1r) took up 20% more antibody than did the cells of her father, which were of the same Rh phenotype (Rh_1) and zygosity.

The quantity of antibody bound to the red cells of unrelated $Rh_0(D)$ homozygous individuals of the same Rh phenotype (Rh_1) showed an almost sixfold variation. A consequence of this observation was that the cells of $Rh_0(D)$ heterozygous children of high antibody uptake fathers took up more antibody than did the cells of low antibody uptake $Rh_0(D)$ homozygous fathers. The gene dosage effect for the $Rh_0(D)$ antigen demonstrable within a family does not appear to apply when unrelated individuals are tested, even though they may be of the same Rh phenotype.

Introduction

Many serological and isotopic studies (1–5) have shown that a complex relationship exists between the red cell $Rh_0(D)$ antigen content and the donor's genotype. Conventional serological techniques are unable to discriminate between $Rh_0(D)$ heterozygous and homozygous red cells except for a few unusual red cell types such as the (D − −) cell (6, 7).

In a previous study (3) $Rh_0(D)$–positive red cells obtained from a blood bank donor population

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were tested with ^{131}I–labeled anti-Rh_0(D). The frequency distribution of the amount of anti-Rh_0(D) bound to the red cells derived from this population was analyzed. Only those Rh_0(D)–positive red cell types that did not contain the rh'(C) antigenic determinant segregated into two populations. There was a 1.6–fold difference between the means of the two populations, suggesting that the two red cell populations represented the Rh_0(D) heterozygous and homozygous states. Red cells containing the rh'(C) antigenic determinant did not show the expected bimodal distribution.

The improvement in techniques that has occurred since the previous report and the identification of some of the factors (8, 9) that affect the reaction between the red cell and the anti-Rh_0(D) encouraged us to re-examine the relationship between genotype and red cell Rh_0(D) antigen content.

The present study differs from the previous report (3) in a number of important respects. The red cells examined in this study were obtained from the members of families in which there was a history of hemolytic disease of the newborn due to fetomaternal Rh_0(D) incompatibility. In such families the Rh_0(D) heterozygous state was represented by the Rh_0(D)–positive red cells of the children. We chose these families to ascertain the heterozygous state and not to study the factors involved in producing erythroblastosis.

Each sample of red cells was tested with different concentrations of antibody so that the optimal proportion of cells to antibody was experimentally determined. Previously (3) the cells were tested at only one antibody concentration, which, for some cells, may have been insufficient to establish equilibrium and, for other cells, may have been in sufficient antibody excess to produce inhibition of antibody binding (10). The antibody bound to the red cells is presented in terms of red cell numbers rather than, as previously, in terms of red cell volume determined by centrifugation. Presentation of the results in this form avoids introducing into the data any differences that may be due to variations in the red cell volumes among the different donors. Finally, the ^{125}I–labeled anti-Rh_0(D) used in the present study was derived from a gamma G fraction obtained by DEAE chromatography rather than from a crude gamma G fraction contaminated with other plasma proteins (11).

The Rh_0(D) red cell antigen content of 63 members of 11 families was examined with ^{125}I–labeled antibody. The relationship between the Rh_0(D) content of the children's cells and that of the father as well as that among the children was examined.

Methods

Family data. Eleven families were studied. The red cells of 41 children, 11 fathers, and 11 mothers were tested. Of the 63 red cells tested in these families, 49 were Rh_0(D)–positive and 14 were Rh_0(D)–negative. All the 11 fathers were Rh_0(D)–positive, all the mothers Rh_0(D)–negative, and, of the 41 children, 3 were Rh_0(D)–negative. The probable Rh genotypes[1] of the 49 (Rh_0(D)–positive red cells included: 29 *R^1r* (CDe/cde), 6 *R^1R^1* (CDe/CDe), 6 *R^0r* (cDe/cde), 3 *R^2r* (cDE/cde), 2 *R^1r'* (CDe/Cde), 1 *R^0R^0* (cDe/cDe), 1 *R^1R^2* (CDe/cDE), and 1 *R^2R^2* (cDE/cDE). The probable Rh genotypes of the 11 fathers included above were 6 *R^1R^1*, 2 *R^1r*, and 1 each of *R^0R^0*, *R^1R^2*, and *R^2R^2*. All the families were Caucasian except family Br, which was Negro.

Fourteen of the 41 children were treated by exchange transfusion one or more times. In 6 of the 11 families there were fetal or neonatal deaths due to Rh incompatibility. The children in 2 families were only mildly affected and did not require exchange transfusions.

The family data are summarized in Table I. The probable Rh genotype of the members of these families was assigned on the basis of the family data (unequivocally in those families with Rh_0(D)–negative children).

The isotopic methods described previously (3, 13) were used except for the procedures described below.

Fraction of the anti-Rh_0(D) serum. REZ serum was obtained from a 34–year–old group O, rr Caucasian housewife who delivered an O, R^1r (CDe/cde) infant with hemolytic disease in 1954. She had a Fallopian tube ligation and subsequently was reimmunized on four occasions between 1961 and 1964 by intravenous administration of 2 ml of whole blood from two different O, R^0 (cDe) donors.

Three different globulin preparations obtained from her serum were used for these studies. Globulin preparation A-79 was obtained by ethodin (Rivanol) fractionation (14) and preparations A-87 and A-88 by DEAE chromatography using the technique of Levy and Sober (15). The globulin preparations were trace labeled with ^{125}I using iodine monochloride (16).

[1] The results are presented as probable Rh genotypes (in italics) rather than phenotypes. The genotype assignment is based on the family data. As will be evident from the results, the data obtained in this study do not favor or support any of the three Rh nomenclatures in current use (6, 7, 12).

TABLE I

Family data

Family	Family member	Age	Probable Rh genotype	Severity of erythroblastosis fetalis*	Preparation used
		years			
Mo	Fa	25	O, R^1R^1		A-79
	Mo	25	O, rr		
	D-1†	6	O, R^1r	N	
	S-2†	4	O, R^1r	S-EX·1	
Br‡	Fa	32	A, R^0R^0		A-87
	Mo	30	O, rr		
	S-1	11	O, R^0r	N	
	D-2	8	O, R^0r	N	
	S-3	6	O, R^0r	S-EX·1	
	S-4	5	A, R^0r	S-EX·3	
	D-5	3	A, R^0r	S-EX·1	
	D-6	2	A, R^0r	M	
Pe	Fa	36	O, R^1R^1		A-87
	Mo	35	B, rr		
	D-1	12	B, R^1r	N	
	D-2	9	O, R^1r	N	
	S-3	7	O, R^1r	N	
	D-4	6	B, R^1r	S-EX·1	
	S-5	4	O, R^1r	S-EX·1	
	D-6	2	B, R^1r	S-EX·1	
Sh	Fa	38	O, R^1R^1		A-87
	Mo	32	O, rr		
	S-1	9	O, R^1r	N	
	S-2	8	O, R^1r	N	
	D-3	7	O, R^1r	M	
	D-4	6	O, R^1r	M	
	S-5	2	O, R^1r	S-EX·1	
Di	Fa	34	A, R^1r		A-87
	Mo	34	A, rr		
	D-1	11	A, R^1r	N	
	D-2	7	A, rr	N	
	D-3	5	A, R^1r	M	
	S-4	4	A, R^1r	S-EX·1	
	D-5	2	A, R^1r	S-EX·1	
Go	Fa	34	O, R^1R^1		A-87§
	Mo	29	A, rr		
	S-1	7	A, R^1r	N	
	S-2	3	A, R^1r	S-EX·3	
	D-3	1	A, R^1r	S-EX·2	
Th	Fa	41	A, R^1r		A-87§
	Mo	34	O, rr		
	S-1	6	A, R^1r	N	
	D-2	5	A, R^1r	N	
	S-3	4	A, rr	N	
	S-4	2	A, R^1r	M	
	S-5	1	A, rr	N	
Is	Fa	26	O, R^1R^2		A-87
	Mo	24	O, rr		
	S-1	7	O, R^1r	N	
	S-2	3	O, R^2r	N	
	D-3	2	O, R^2r	M	
Co	Fa	30	O, R^1R^1		A-88
	Mo	28	A, rr		
	S-1	7	O, R^1r	N	
	S-2	6	O, R^1r	N	
	S-3	3	O, R^1r	N	
	S-4‖	0.1	$Rh_0(D)$ +	S-EX·4	
Ma	Fa	35	A, R^2R^2		A-88
	Mo	34	A, rr		
	D-1	15	O, R^2r	N	
Re	Fa	32	O, R^1R^1		A-88
	Mo	27	A, $r'r$		
	D-1	9	A, R^1r'	N	
	D-2	9	A, R^1r'	M-EX·1	

* Severity of erythroblastosis fetalis indicated by the following code: N = normal; M = mild, no exchange; S = severe; EX·1 = exchanged once, and so forth.
† D = daughter; S = son.
‡ Negro family; all others Caucasian.
§ Acid eluates; all others were heat eluates.
‖ Not tested.

Radioautography of the immunodiffusion and immunoelectrophoresis patterns of ethodin globulin preparation A-79 showed that gamma A, gamma M, transferrin, and albumin, in addition to gamma G, were present. All the ^{125}I in the DEAE globulin preparations, A-87 and A-88, was associated with the gamma G protein. The properties of the ^{125}I–labeled anti-D–containing globulin fractions are shown in Table II.

Preparation of eluates containing ^{125}I–labeled-$Rh_0(D)$. The heat eluate derived from preparation A-79 was obtained by sensitizing a pool of lyophilized D–positive red cell stromata with the ^{125}I–labeled globulin fraction at 37° C for 90 minutes. The sensitized stromata were washed four times and eluted by using the techniques described previously (3, 13). A total of 80 g of stromata derived from a pool consisting of 34 U of $Rh_0(D)$–positive red cells was used, and 0.31 mg globulin nitrogen per g of lyophilized stromata was used for sensitization.

The eluates obtained from the gamma G–^{125}I preparations A-87 and A-88 were derived from sensitized intact red cells. A pool composed of 16 U of R^1 (CDe) and R^2 (cDE) red cells was used for A-87, and a 9–U pool was used for A-88. The red cells were sensitized with the gamma G–^{125}I at 37° C for 90 minutes by using 2.78 mg A-87 globulin nitrogen and 2.81 mg A-88 globulin nitrogen per 200 ml of packed red cells. The sensitized cells were washed and converted to stromata by freezing and thawing three times after the addition of 1 vol of buffered isotonic saline, pH 6.5, containing 2% bovine serum albumin (BSA). The stromata were recovered by centrifugation in the Spinco model L at 73,000 maximal *g* for 15 minutes and washed by centrifugation four times with buffered isotonic saline, pH 6.5, containing 0.1% BSA.

The washed sensitized stromata derived from preparation A-87 were divided into two equal portions. One was eluted with heat, 56° C for 20 minutes, using pH 5.8, 0.15 M phosphate buffer containing 0.03% carrier human gamma G. The other portion of sensitized stromata as well as the A-88 sensitized stromata was eluted with acid. The stromata were suspended in saline containing 2% BSA, and the pH was gradually lowered at 23° C to pH 3.2 using 0.2 N HCl. After adjustment of the pH, the supernatant containing the eluted ^{125}I–labeled anti-$Rh_0(D)$ was recovered by centrifugation and adjusted to pH 6.5 with 0.2 N NaOH. The anti-$Rh_0(D)$ was exposed to pH 3.2 for 20 to 30 minutes by this technique.

All eluates were concentrated six– to eightfold with polyethylene glycol (Carbowax), and the concentrated eluates were readjusted to pH 6.5 by dialysis against buffered isotonic saline, pH 6.5, $\mu = 0.16$. The nitrogen content of the four anti-$Rh_0(D)$–containing eluates ranged from 0.571 to 0.080 μg nitrogen per ml. The eluate nitrogen was calculated from the ^{125}I content of the eluates and the ^{125}I to nitrogen ratio of the anti-$Rh_0(D)$–containing gamma G fraction. The nitrogen content of the gamma G fraction was determined by the micro-Kjeldahl method (17). The anti-$Rh_0(D)$ titers with the antiglobulin reaction ranged from 16 to 32. The original REZ serum had an anti-rh′ (C) titer of 1:8, but the eluates were

TABLE II

*Properties of ^{125}I-labeled anti-$Rh_0(D)$-containing globulin fractions and eluates**

	A-79		A-87			A-88	
	Globulin	Heat eluate	Globulin	Heat eluate	Acid eluate	Globulin	Acid eluate
Antiserum donor	REZ		REZ			REZ	
Mg N iodinated	29.2		21.7			20.0	
Per cent ^{125}I bound	63.4		63.7			65.5	
Moles I per mole globulin†	3.3		3.0			3.4	
Per cent ^{125}I TCA precipitable‡	99.6	98.8	96.9	99.2	98.9	97.9	99.2
Nitrogen, μg/ml	674	0.571	551	0.094	0.080	469	0.147
μc ^{125}I/μg N§	1.41		2.11			3.12	
Anti-$Rh_0(D)$ titers‖	256	32	256	16	32	512	32
Per cent ^{125}I absorbed by $Rh_0(D)$-positive cells		21.0		75.7	80.5		83.9
Per cent ^{125}I absorbed by $Rh_0(D)$-negative cells		0.02		0.60	1.23		1.0

* Globulin fraction A-79 was obtained by ethodin fractionation (14), whereas globulin fractions A-87 and A-88 were obtained by DEAE chromatography (15). Heat eluates were prepared by heating the sensitized stromata to 56° C for 20 minutes at pH 5.8 and acid eluates by adjusting the pH to 3.2.

† Calculated with a molecular weight of 160,000 for the globulin fraction iodinated.

‡ TCA = trichloroacetic acid.

§ Activity at the time of iodination.

‖ Titers were determined with the indirect antiglobulin method using a commercial antiserum and group O, R^1R^2 cells.

negative for anti-rh′ (C) in both the antiglobulin reaction and isotopically as determined by reaction with r′r (Cde/cde) cells. These data as well as the per cent of ^{125}I absorbable by $Rh_0(D)$-positive and $Rh_0(D)$-negative cells are shown in Table II. The differences in the properties of eluates derived from heat and acid elution of cells or stromata sensitized with either ethodin- or DEAE-prepared gamma G are discussed elsewhere (18).

Reaction of red cells with ^{125}I-labeled anti-$Rh_0(D)$. Red cell suspensions, usually 10%, were prepared by using the microhematocrit technique (19). A 1% suspension by volume was used for the reaction with the antibody. The number of red cells present in this dilution was determined by electronic counting with the Coulter counter (20, 21). In a typical study, 0.1 ml of a 1% red cell sample (about 10^7 cells) was incubated with different amounts of ^{125}I-labeled anti-$Rh_0(D)$ at 37° C for 60 minutes. After reaction the sensitized cells were washed four times with cold phosphate-buffered saline, pH 6.5, $\mu = 0.16$. There was minimal dissociation of cell-bound ^{125}I during washing. Dissociation of 10 to 15% of the cell-bound ^{125}I was possible only after incubation at 37° C for 1 hour. The quantity of ^{125}I bound to the red cells was determined by well-type scintillation counting. Ionic strength was adjusted by diluting the mixture of red cells and anti-$Rh_0(D)$ at pH 6.5 with 0.29 M glycine.

Determination of radioactivity. The ^{125}I content of the different samples was measured by gamma ray spectrometry with a well-type scintillation detector ($1\frac{3}{4}$- × 2-inch thallium-activated NaI crystal) and an automatic sample changer (Packard). Counting of the 7.4 and 35.4 kev ^{125}I photopeaks was carried out with a 55-kev window centered at 30 kev. With this mode of counting, 72% of the total ^{125}I disintegrations were detected, and the background counting rate was 50 to 60 cpm. All samples containing ^{125}I were placed in thin-walled cellulose nitrate tubes ($\frac{1}{2}$ × $2\frac{1}{2}$ inches, 43.4 mg per cm^2) instead of glass to reduce the absorption of the low energy photons by the tube. The sample tube was then placed into another tube for automatic counting ($\frac{17}{32}$ × 6 inches, 44.18 mg per cm^2). Unless indicated in the tables, all samples had counting rates greater than 10 times background. The coefficient of variation for replicate determinations with the same red cell suspension was less than 1%. There was a 5 to 10% variation when a fresh sample was tested on different days.

Calculations. The evidence available (22–24) indicates that the reaction between red cells and anti-$Rh_0(D)$ is reversible and conforms to the law of mass action. The data are presented both as cell-bound nitrogen at equilibrium and as values extrapolated with the Scatchard equation (25), as was done by Hughes-Jones and others (24–26). The equilibrium value represents the amount of cell-bound anti-$Rh_0(D)$ under the conditions of reaction used for testing the cells; pH, ionic strength, time, temperature, proportion of cells to antibody, and so forth. The cell-bound nitrogen under the conditions of reaction used in this study (equilibrium value) was about 80% of the extrapolated value. The assumptions involved in converting radioactivity to nitrogen have been discussed previously (3, 11, 13).

We facilitated data processing by transcribing the data from the automatic counter directly onto IBM cards by coupling an IBM 526 card punch summary to the counter. The data on the IBM cards were processed by using an IBM 1620 or 1401 computer and a program designed for these studies.

Results

Cell-bound ^{125}I-labeled anti-$Rh_0(D)$ at equilibrium. The effect of progressively increasing the

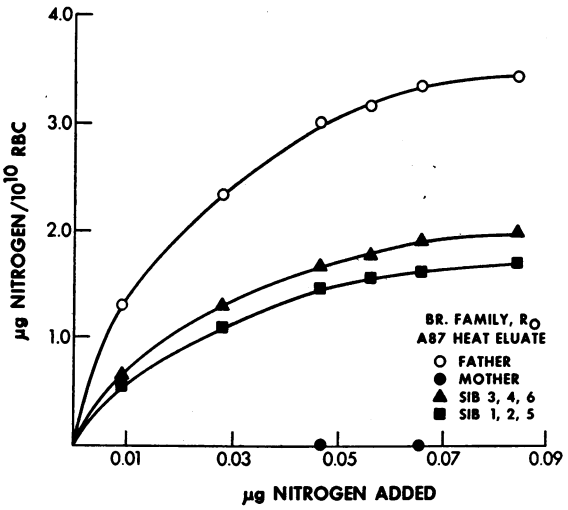

FIG. 1. RELATIONSHIP BETWEEN NITROGEN ADDED AND QUANTITY OF NITROGEN BOUND TO RED CELLS OF FAMILY MEMBERS. Preparation A-87, heat eluate. Reaction was carried out at pH 6.5 and $\mu = 0.16$. Total reaction volume was 1.0 ml with about 9×10^6 red cells. The values were so similar for the six children that only two curves are required to present the data. RBC = red blood cells.

amount of added eluate nitrogen on the anti-$Rh_0(D)$ bound to a constant number of red cells is shown for three different families in Figures 1, 2, and 3. As the nitrogen added is increased, there is a progressive increase in the nitrogen bound until a plateau is reached. With the ^{125}I–labeled anti-$Rh_0(D)$ used for these studies, the maximal quantity of cell–bound antibody occurred when less than 5% of the added nitrogen was cell–bound, indicating that only in antibody excess was maximal uptake obtained. There was no marked inhibition of antibody uptake in antibody excess with the degree of antibody excess used in this study.

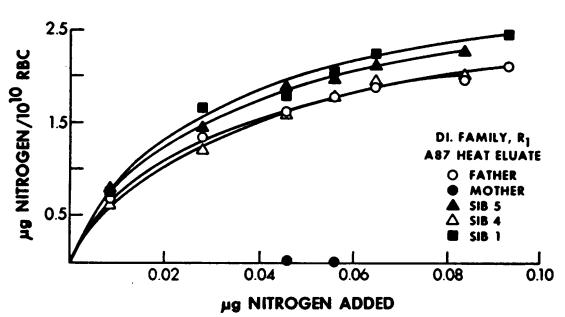

FIG. 2. RELATIONSHIP BETWEEN NITROGEN ADDED AND QUANTITY OF NITROGEN BOUND TO RED CELLS OF FAMILY MEMBERS. Preparation A-87, heat eluate. The reaction was carried out at pH 6.5 and $\mu = 0.16$. Total reaction volume was 1.0 ml with about 9×10^6 red cells. Data for the father, mother, and only three of the five children are shown.

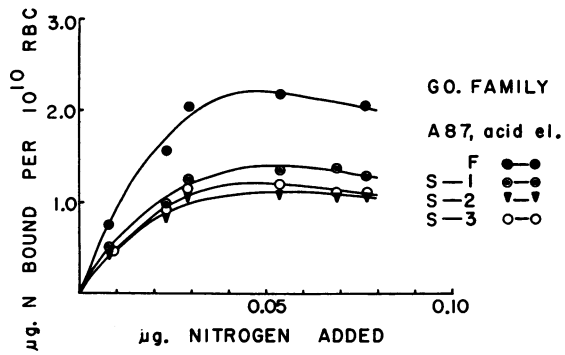

FIG. 3. RELATIONSHIP BETWEEN NITROGEN ADDED AND QUANTITY OF NITROGEN BOUND TO RED CELLS OF FAMILY MEMBERS. Preparation A-87, acid eluate. Reaction was carried out at pH 6.5 and $\mu = 0.16$. Total reaction volume was 1.1 ml with about 9×10^6 red cells.

Two types of family red cell uptake curves are evident in the figures. In one pattern, shown by homozygous fathers such as in the Br family in Figure 1 and the Go family in Figure 3, the anti-$Rh_0(D)$ bound to the father's red cells is about twice that bound to his children's cells. In the other pattern, found with heterozygous fathers as in the Di family in Figure 2, about the same quantity of antibody is bound to the red cells of the father and his children. The relationship between the father and his children with respect to antibody uptake appears to be maintained at all concentrations of added eluate nitrogen (Figures 1 and 3).

The data for all the red cells tested in this study were graphically analyzed by using the Scatchard equation, as in Figure 4, to obtain an estimate of the total antibody required to saturate all of the antigenic sites. With the conditions used in this study, about 75 to 80% of the total red cell sites appear to be occupied at equilibrium (Table IV).

Reactivity of acid and heat anti-$Rh_0(D)$ eluates. Seven families were studied with both acid and heat eluates derived from preparation A-87. The antibody taken up from the acid eluate by the 22 $Rh_0(D)$–positive individuals in four families averaged about 91% of the antibody taken up from the heat eluate. This small difference between the heat and acid eluates was independent of the

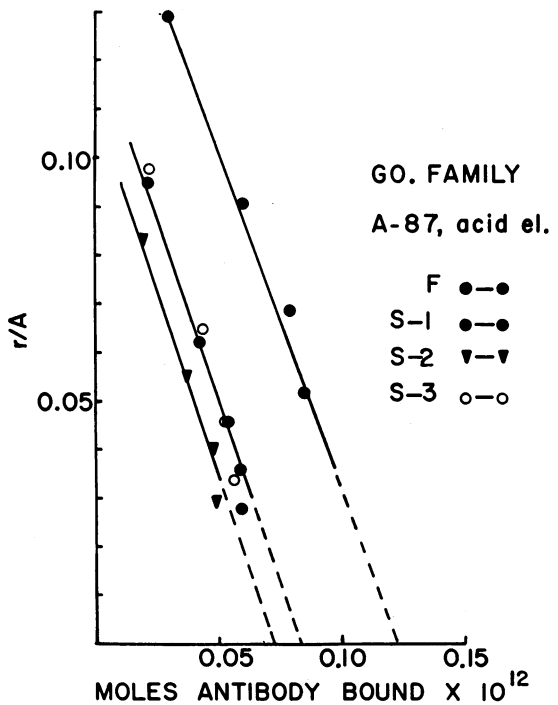

FIG. 4. RELATIONSHIP BETWEEN MOLES OF CELL-BOUND ANTI-Rh_0(D) (r) TO THE RATIO OF CELL-BOUND TO FREE ANTI-Rh_0(D) (r/A). The data shown in Figure 3 were used to obtain this plot. A represents free anti-Rh_0(D).

Rh genotype, so that the relationship between the father and his children with respect to antibody uptake was maintained irrespective of the type of eluate used.

Anti-Rh_0(D) uptake as a function of red cell numbers and volume. The cell-bound anti-Rh_0(D) was expressed both on the basis of red cell numbers and red cell volume. The ratio between red cell numbers and volume varied from 0.82 to 1.22 and indicates that the two methods of expressing the results are not equivalent. If the Rh_0(D) antigen content is a function of individual red cells and independent of the red cell surface area, then the best estimate of the red cell Rh_0(D) content will be obtained by using red cell numbers. If the antigen content is a function of surface area, then there would be an inverse relationship between antigen content and mean corpuscular volume. The data, however, cannot be used to test this interpretation because there is volume heterogeneity due to red cell age. A clear-cut answer to this problem must await measurements of antibody uptake of individual cells.

Effect of ionic strength on the red cell binding of ^{125}I-labeled anti-Rh_0(D). The data in Table III show that when the ionic strength of the reaction medium was reduced from 0.26 to 0.12, there was about a 2.5-fold increase in the equilibrium value of cell-bound anti-Rh_0(D). The increased binding at low ionic strength was proportional to the binding at high ionic strength, so that the relationship between the father's red cells and those of his children was maintained at either ionic strength (see last two columns, Table III).

Results obtained with different ^{125}I-labeled anti-Rh_0(D)-containing eluates. Members of the Br family were tested on different dates with two heat eluates derived from two different globulin preparations, A-79 and A-87. The two preparations differed in many major respects (see Table II). Globulin fraction A-79 was a crude gamma G obtained by ethodin fractionation contaminated with albumin, transferrin, and other proteins. Globulin fraction A-87 was a homogeneous gamma G preparation obtained by DEAE chromatography. The A-79 eluate was obtained after sensitization of lyophilized stromata, whereas the A-87 eluate was obtained after sensitization of fresh intact red cells. In spite of these differences in the preparation of the two eluates, the relationship between the father and his children with respect to quantity of cell-bound anti-Rh_0(D) was al-

TABLE III

*Effect of ionic strength on the binding of ^{125}I-labeled anti-Rh_0(D) to the red cells of family members**

Family member	Age	Probable Rh genotype	N bound to 10^{10} RBC†		Fraction of father	
			μ=0.26	μ=0.12	μ=0.26	μ=0.12
	years		μg			
Mo family						
Fa	25	O, R^1R^1	2.58	6.27	1.00	1.00
Mo	25	O, *rr*	0.23	0.33	0.99	0.05
D-1	6	O, R^1r	1.35	3.35	0.52	0.53
S-2	4	O, R^1r	1.50	3.48	0.53	0.56
Br family‡						
Fa	32	A, R^0R^0	2.34	4.90	1.00	1.00
Mo	30	O, *rr*	0.22		0.09	
S-3	6	O, R^0r	1.27	2.89	0.54	0.59
S-4	5	A, R^0r	1.25	2.93	0.54	0.60
D-5	3	A, R^0r	0.94	2.33	0.40	0.48

* Preparation A-79. Values represent red cell uptake of ^{125}I as the eluate nitrogen added was progressively increased. About 5×10^7 red cells was used for the reaction, and the total reaction volume was 1.45 ml (Mo family) and 1.65 ml (Br family) at μ=0.26. Ionic strength was adjusted by the addition of 0.29 M glycine at pH 6.5, and total reaction volume at low ionic strength (0.12) was 2.45 ml (Mo family) and 2.65 ml (Br family).
† RBC =red blood cells.
‡ Negro family.

TABLE IV

Quantity of ^{125}I-labeled anti-D bound to the red cells of family members

Family member	Probable Rh genotype	N bound at equilibrium	Ratio of equilibrium N to extrapolated N*	Molecules per cell from extrapolated N	Fraction of father	
					N value at equilibrium	Extrapolated N value
		$\mu g\ N/10^{10}$ RBC				
Mo family†						
Fa	O, R^1R^1	2.58	0.60	10,500	1.00	1.00
Mo	O, rr	0.23			0.09	
D-1	O, R^1r	1.35	0.58	6,070	0.55	0.58
S-2	O, R^1r	1.50	0.61	5,160	0.58	0.49
Br family‡						
Fa	A, R^0R^0	3.47	0.78	10,500	1.00	1.00
Mo	O, rr	0.07			0.02	
S-1	O, R^0r	1.74	0.79	5,180	0.54	0.49
D-2	O, R^0r	1.74	0.81	5,050	0.54	0.48
S-3	O, R^0r	2.03	0.82	5,750	0.58	0.55
S-4	A, R^0r	2.09	0.81	6,020	0.60	0.57
D-5	A, R^0r	1.71	0.80	5,040	0.49	0.48
D-6	A, R^0r	1.98	0.81	5,760	0.57	0.55
Pe family§						
Fa	O, R^1R^1	2.00	0.75	6,220	1.00	1.00
Mo	B, rr	0.04			0.02	
D-1	B, R^1r	1.15	0.78	3,500	0.57	0.57
D-2	O, R^1r	1.65	0.78	4,960	0.83	0.80
S-3	O, R^1r	1.31	0.73	4,220	0.66	0.68
D-4	B, R^1r	1.61	0.78	4,850	0.81	0.78
S-5	O, R^1r	1.75	0.78	5,300	0.87	0.85
D-6	B, R^1r	1.64	0.74	4,410	0.82	0.71
Sh family‡						
Fa	O, R^1R^1	3.30	0.75	10,380	1.00	1.00
Mo	O, rr	0.06			0.02	
S-1	O, R^1r	1.86	0.76	5,170	0.56	0.50
S-2	O, R^1r	2.01	0.76	5,480	0.61	0.53
D-3	O, R^1r	2.50	0.77	6,760	0.75	0.65
D-4	O, R^1r	2.12	0.80	5,690	0.64	0.55
S-5	O, R^1r	2.38	0.75	6,900	0.72	0.67
Di family‡						
Fa	A, R^1r	1.97	0.78	5,970	1.00	1.00
Mo	A, rr	0.10			0.10	
D-1	A, R^1r	2.46	0.85	6,780	1.25	1.14
D-2	A, rr	0.23			0.12	
D-3	A, R^1r	2.06	0.80	6,050	1.05	1.01
S-4	A, R^1r	1.97	0.79	5,860	1.00	0.98
D-5	A, R^1r	2.26	0.80	6,630	1.15	1.11
Go Family§						
Fa	O, R^1R^1	2.20	0.75	7,500	1.00	1.00
Mo	A, rr	0.05			0.02	
S-1	A, R^1r	1.39	0.71	4,600	0.63	0.61
S-2	A, R^1r	1.09	0.68	3,850	0.50	0.51
D-3	A, R^1r	1.20	0.68	4,300	0.56	0.57

* Ratio of the maximal nitrogen bound to the red cells over the value derived with the Scatchard equation (25).

† Preparation A-79, heat eluate obtained from sensitized stromata, total volume 1.45 ml, $\mu = 0.26$, about 4.5×10^7 RBC per reaction tube, and maximal nitrogen added = 0.80 μg.

‡ Preparation A-87, heat eluate obtained from sensitized red cells, total volume 1.0 ml, $\mu = 0.16$, about 9×10^6 RBC per reaction tube, and maximal nitrogen added = 0.084 μg.

§ Preparation A-87, acid eluate obtained from sensitized red cells, total volume 1.2 ml, $\mu = 0.16$, about 9×10^6 RBC per reaction tube, and maximal nitrogen added = 0.077 μg.

‖ Preparation A-88, acid eluate obtained from sensitized red cells, total volume 2.1 ml, $\mu = 0.16$, about 9×10^6 RBC per reaction tube, and maximal nitrogen added = 0.218 μg.

TABLE IV—(*Continued*)

Family member	Probable Rh genotype	N bound at equilibrium	Ratio of equilibrium to extrapolated N*	Molecules per cell from extrapolated N	Fraction of father	
					N value at equilibrium	Extrapolated N value
		$\mu g\ N/10^{10}$ RBC				
Th family§						
Fa	A, R^1r	1.76	0.70	5,150	1.00	1.00
Mo	O, rr	0.03			0.02	
S-1	A, R^1r	1.61	0.75	4,920	0.92	0.96
D-2	A, R^1r	1.90	0.84	5,310	1.08	1.03
S-3	A, rr	0.04			0.02	
S-4	A, R^1r	1.67	0.84	4,700	0.95	0.91
S-5	A, rr	0.03			0.02	
Is family‡						
Fa	O, R^1R^2	3.80	0.71	12,700	1.00	1.00
Mo	O, rr	0.09			0.02	
S-1	O, R^1r	2.04	0.79	6,090	0.54	0.48
S-2	O, R^2r	2.55	0.78	7,700	0.67	0.61
D-3	O, R^2r	2.97	0.81	8,220	0.78	0.65
Co family‖						
Fa	O, R^1R^1	2.43	0.78	7,340	1.00	1.00
Mo	A, rr	0.12			0.05	
S-1	O, R^1r	1.94	0.78	5,910	0.80	0.81
S-2	O, R^1r	1.75	0.76	5,560	0.72	0.76
S-3	O, R^1r	1.72	0.76	5,370	0.71	0.73
Ma family‖						
Fa	A, R^2R^2	4.30	0.77	13,030	1.00	1.00
Mo	A, rr	0.17			0.04	
D-1	O, R^2r	2.71	0.80	8,000	0.63	0.61
Re family‖						
Fa	O, R^1R^1	11.94	0.77	36,700	1.00	1.00
Mo	A, $r'r$	0.12			0.01	
D-1	A, R^1r'	7.95	0.75	24,820	0.67	0.68
D-2	A, R^1r'	7.99	0.76	24,750	0.67	0.67

most identical for the two eluates. The nitrogen values obtained with preparation A-79 were from 55 to 68% of those obtained with preparation A-87. This difference in large part was due to differences in ionic strength. Tests with A-79 were carried out at $\mu = 0.26$, whereas the tests with A-87 were carried out at $\mu = 0.16$.

Quantity of ^{125}I–labeled anti-$Rh_0(D)$ bound to the red cells of family members. The data for all 11 families are summarized in Table IV. The Table shows the cell–bound nitrogen at equilibrium, the ratio of this value to the extrapolated nitrogen value, and the number of molecules per cell calculated by using the extrapolated value. The last two columns present the cell–bound nitrogen of family members as a ratio of the father's, using both equilibrium and extrapolated nitrogen values.

The 14 D–negative red cells took up from 1 to 10% as much of the ^{125}I–labeled anti-$Rh_0(D)$ as was bound to the fathers' cells at equilibrium, whereas the $Rh_0(D)$–positive cells of the children in these families took up from 49 to 125% of the anti-$Rh_0(D)$ bound to the fathers' cells.

The cell–bound nitrogen at equilibrium for preparations A-87 and A-88 was 71 to 88% of the nitrogen value obtained by extrapolation, and 60% for A-79. The nitrogen ratios among the father and his children obtained by using the equilibrium value were almost identical to those found with the extrapolated value.

To facilitate comparisons within and between families, the data are presented by probable Rh genotype in Table V. The results are shown as average number of $Rh_0(D)$ antigen sites per red cell, assuming that one antibody molecule binds to ane antigen site. The zygosity of all the fathers can be assigned unequivocally when the red cells of

TABLE V

*Number of Rh$_0$(D) antigen sites among the red cells of family members**

Number of sites × 10^3 (extrapolated values)								
R^1r	R^1R^1	R^1r'	R^0r	R^0R^0	R^1r	R^1R^2	R^2r	R^2R^2
6.07	10.50[a]		5.92	9.95[b]			8.00	13.03
5.16			5.25					
			4.72					
			5.40		5.87	11.00[c]	5.91	
3.50	6.22[d]		4.76				7.23	
4.96			4.55					
4.22								
4.85								
5.30								
4.41								
5.17	10.38[f]							
5.48								
6.76								
5.69								
6.90								
5.71[g]								
6.19								
5.55								
5.98								
6.84								
4.60	7.50[h]							
3.85								
4.30								
5.15[i]								
4.92								
5.31								
4.70								
5.91	7.34[j]							
5.56								
5.37								
	36.70[k]	24.82						
		24.75						
Totals = 28	6	2	6	1	1	1	3	1

[a] = Mo family, A-79; [b] = Br family, A-87; [c] = Ma family, A-88; [d] = Pe family, A-87; [e] = Is family, A-87; [f] = Sh family, A-87; [g] = Di family, A-87; [h] = Go family, A-87; [i] = Th family, A-87; [j] = Co family, A-88; [k] = Re family, A-88. All acid eluates except Mo family. Father's value is underlined.

the father are tested simultanously with those of his children. Of the 11 fathers, 2 were heterozygous R^1r, and 9 were homozygous (6 R^1R^1, 1 R^0R^0, 1 R^1R^2, and 1 R^2R^2). No heterozygous children bound more than 85% of the anti-Rh$_0$(D) taken up by the 9 homozygous fathers. The 31 children of the homozygous fathers took up an average of 62% of the antibody bound to their fathers' red cells.

The two heterozygous fathers were readily identified because the red cells of their 7 children took up, on the average, 103% of the anti-Rh$_0$(D) bound to the fathers' cells (range 91 to 120%). The assignment of the heterozygous state was confirmed independently of the isotopic data by the presence of Rh$_0$(D)–negative children in these two families.

There was at least a twofold variation in the quantity of Rh$_0$(D) antigen sites on the red cells of the homozygous R^1R^1 fathers. The R^1R^1 father in the Pe family had 6,220 sites per cell, whereas the R^1R^1 father in the Sh family had 10,380 Rh$_0$(D) sites. If the unusually high in uptake Re family is included, there is an almost sixfold range in red cell Rh$_0$(D) antigen sites on the R^1R^1 cell, from 6,200 to 36,700. It is unlikely that the high values in the Re family are due to experimental error because each person was tested at four different concentrations of antibody and all members of the family showed the same high uptake.

Discussion

It is generally recognized that the serology and genetics of the Rh$_0$(D) red cell antigen are exceedingly complex and poorly understood (6, 7). There is reason to believe that the Rh$_0$(D) antigen includes a spectrum of antigenic determinants. The antisera in use, therefore, are probably polyvalent and of different specificities, depending on the Rh$_0$(D) antigens involved in the immunization of the donor. The difficulties associated with the Rh$_0$(D) antigen in large measure are due to the fact that the antigen consists of an unknown number of specificities and the antisera used for the study of the antigen consist of unknown mixtures of antibodies to different Rh$_0$(D) determinants. Hirschfeld (27) has discussed the problems associated with such an immunogenetic system and has appropriately described it as a complex–complex system, i.e., an antigen containing multiple determinants and antisera with many specificities. Unfortunately, our understanding of the Rh$_0$(D) antigen is handicapped further by the paucity of information on the chemical nature of this antigen (28).

The present study has used iodine-labeled anti-Rh$_0$(D) to study the quantity of Rh$_0$(D) antigen on the red cells of family members. Before considering the significance of the results obtained it may be useful to review some of the limitations associated with the use of this technique. An obvious one is that this method can only measure the antigenic determinants that are represented by an-

tibodies in the serum of our donor, REZ. In addition, these antibodies may not be equally represented in the antibodies eluted. The results obtained by Rosenfield, Szymanski, and Kochwa (29), who showed that the first and second absorption anti-Rh_0(D) eluates have different capacities to fix to the same cell, suggest that elution may select different Rh_0(D) specificities.

The families included in the study were not selected at random. They were identified and included in the study because there had been a history of clinically significant hemolytic disease of the newborn due to Rh incompatibility. The degree of selection involved in using such a population is evident from the fact that only 2 to 5% of Rh–incompatible pregnancies result in hemolytic disease (30). Consequently, the families used for this study may differ from the general population with respect to the Rh_0(D) antigen. The families also differed among themselves with respect to another factor involved in maternal Rh sensitization. There is good evidence to indicate that fetomaternal incompatibility in the ABO blood group system protects against primary maternal Rh sensitization (31, 32). Two families in the present study had such fetomaternal incompatibility in the ABO antigens. The type O mothers in the Br and Th families had type A children. Only one of the A children in the Th family had mild disease, since all the children were A and incompatible with their O mother. The first three children in the Br family, however, were O and compatible with their O mother, so that the mother was sensitized to the Rh_0(D) antigen. The ABO incompatibility of the last three A children did not protect the children in the Br family because the mother had been sensitized by previous ABO–compatible pregnancies. There was no evidence that these two families with ABO incompatibility were different from the other families with respect to the amount of red cell–bound anti-Rh_0(D).

The antigenic strength of the Rh_0(D) antigen of fetal cells has been implicated as an important factor in producing hemolytic disease (33). If clinical disease in the families studied was due to a greater antigenicity of the Rh_0(D) antigen, then the data obtained with the Rh_0(D) antigen found in these families may not apply to the Rh_0(D) antigen found in the general population. An insufficient number of families was available to determine if this may be the case.

Another factor to be considered is the disparity in age involved in comparing the red cells of the children with those of the fathers. The average age of the 11 fathers was 33 years (range from 25 to 41), whereas the average age of the 38 Rh_0(D)–positive children was 5.8 years (range from 1 to 15). Comparisons between these two groups would not be valid if the Rh_0(D) red cell antigen is affected by the age of the donor. Grundbacher (34) has shown that the A_1 antigen increases in strength during the first 4 years. No evidence has been produced in this study to show that the Rh_0(D) antigen changes with the red cell donor's age.

There are three possible ways in which gamma G anti-Rh_0(D) may combine with antigenic determinants on the red cell. The calculation used assumes that the antibody binds to the red cell with only one combining site, so that the number of molecules bound represents the number of antigenic determinants. If both combining sites of the antibody are fixed to the red cell, the number of antigenic sites shown in our data would represent only half of the Rh_0(D) red cell sites. This mode of binding is unlikely unless the Rh_0(D) antigenic sites are grouped together in clusters or patches to allow the relatively short antibody molecule (240 A) to bridge two sites (35). The third possible mode of binding would be with both univalent and bivalent binding on a random basis. If this should be the case, then there would be no simple correspondence between the amount of anti-Rh_0(D) bound to the red cell and the number of antigen sites. Our data do not support this interpretation, since within each family the results were consistent with those to be expected from genetic considerations.

Another assumption implicit in expressing the results as the average number of molecules bound per red cell is that the circulating red cells all contain the same quantity of antigen. Radioautographic studies of the Rh_0(D) antigen content of individual red cells indicate that under certain conditions the circulating red cells from a given donor may not be uniform with respect to their Rh_0(D) antigen content (36). If the degree of heterogeneity in different donors varied, the use of the average value for cell–bound anti-Rh_0(D)

would be unreliable for comparing one donor to another.

The estimates of the $Rh_0(D)$ antigen content of red cells made in the previous report (3) are in agreement with those in the present study. The values ranged from 4,600 to 10,300 $Rh_0(D)$ sites per red cell, whereas in the present study the values varied from 3,500 to 36,700. Only three of the 49 $Rh_0(D)$–positive cells tested in this study had more than 11,000 sites per cell. This agreement between the two studies is fortuitous. The estimates in the earlier study (3) were subject to two errors of equal magnitude but opposite direction, which canceled each other and resulted in estimates that agree with the values found in this study. In the previous study the red cell antigen content was overestimated by using the iodine to nitrogen ratio of a globulin preparation (A-25) that was contaminated with albumin and other proteins differing from gamma G in their tyrosine contents (11). This overestimation was compensated for by the underestimation that resulted from the use of the equilibrium value rather than the quantity of antibody that would be required to occupy all of the antigenic sites.

The values found in the present study are in the range reported by Rosenfield and Kochwa (29, 37), but are less than the estimates obtained by Hughes-Jones and co-workers, who found 24,000 sites per cell with ^{131}I–labeled anti-$Rh_0(D)$ (24) and by Rochna and Hughes-Jones, who found from 10,000 to 33,000 sites per cell with ^{131}I–labeled antigamma globulin (5). In view of the many assumptions used in deriving these values, the values obtained in these different studies are in relatively good agreement.

Evidence was obtained to show that by using ^{125}I–labeled anti-$Rh_0(D)$ under carefully controlled conditions, the dose relationship between the father and his children with respect to red cell antigen content is reproducible under a variety of conditions: ionic strength, different eluates, equilibrium vs. extrapolated values, and so forth. One of the striking findings was that it was possible to assign unequivocally the $Rh_0(D)$ genotype of the father when the antibody uptake of his cells was compared with that of the heterozygous red cells of his children. It was impossible, however, to determine the zygosity of unrelated red cell donors. There was a large overlap between the number of antigen sites on the $Rh_0(D)$ heterozygous and homozygous red cells when unrelated individuals were tested. The heterozygous R^1r child (S–J) of a high in uptake R^1R^1 father in the Sh family had more $Rh_0(D)$ sites (6,900) than did the red cells of the low uptake homozygous R^1R^1 father in the Pe family (6,200). The wide variability in the strength of the $Rh_0(D)$ antigen among unrelated individuals has been noted by others, most recently Greenwalt, Myhre, and Steane (1). The findings in this study differed from theirs in that they were unable to show any difference between the fathers' homozygous R^1R^1 red cells and the heterozygous R^1r red cells of their children. These differences in results are undoubtedly related to differences in the techniques used. These authors used agglutination of enzyme–modified cells, whereas the present study measured the binding of antibody to unagglutinated cells. The hemagglutination technique (38) undoubtedly measures a different end point than the isotopic technique.

The marked variability of the $Rh_0(D)$ content within a given serologically defined Rh phenotype, Rh_1 (CDe), raises many questions. An obvious one concerns the manner in which the phenotype was ascertained. Only the basic Rh phenotype was determined with the five common Rh antisera. It is conceivable that if tests for additional Rh antigens were carried out, such as Rh^A, Rh^B, and so forth (39), these families would fall into different serologically defined Rh phenotypes. An alternative related possibility is that the variability of the same Rh phenotype within these families reflects a deficiency of antibody components in antiserum REZ. If our antiserum did not possess antibodies to all the $Rh_0(D)$ antigenic components, the red cells with a high concentration of these determinants would show a low uptake from this antiserum. The polyvalent nature of antiserum REZ is evident from the fact that it can be fractionated by certain nonhuman primate red cells and by human type D^u red cells (36).

The variability in strength of the $Rh_0(D)$ antigen in different Rh phenotypes is well known (6, 7). There is weakening of the $Rh_0(D)$ antigen when the rh'(C) antigen is present (1–4, 20). This effect is also found when an rh'(C)–positive $Rh_0(D)$–negative allele is paired with an $Rh_0(D)$–carrying allele (40, 41). This effect of the rh'(C)

antigen in the trans position was evident in two families. In the Pe family, the six R^1r children had an average of 73% of the $Rh_0(D)$ antigen found on the father's R^1R^1 cells, and in the Co family, the three R^1r children had an average of 77% of the father's R^1R^1 $Rh_0(D)$ antigen. In both families, the antibody bound to the fathers' cells [double dose of $Rh_0(D)$ antigen] was considerably less than would be expected from the antibody bound to the cells of their children [single dose of $Rh_0(D)$ antigen]. This result would suggest that the presence of the rh'(C) antigen in the trans position is associated with decreased binding of antibody. Three families (Mo, Sh, Go), however, did not show this effect of the rh'(C) in the trans position on the amount of $Rh_0(D)$. The R^1r children in these families took up an average of 54 to 58% of the antibody bound to the R^1R^1 fathers' cells. The Re family also showed no effect of the rh'(C) in the trans position. The two R^1r children took up 68% of the anti-$Rh_0(D)$ bound to their father's R^1R^1 cells. It may be, however, that the father's two R^1 chromosomes are dissimilar and only the high uptake chromosome was transmitted to the two daughters. This family was very unusual because the father and his two daughters took up almost three times as much antibody as the highest value (R^2R^2) found in the other families. There is no obvious explanation for this observation.

The results in these six families clearly show that the presence of rh'(C) antigen on $Rh_0(D)$–positive cells is not consistently associated with a reduction in the amount of $Rh_0(D)$ antigen, as measured with the isotopic technique. It is also evident that there is no consistent effect due to the position of the rh'(C) gene. It would appear that factors other than the association of these two antigens are involved in reducing the quantity of red cell $Rh_0(D)$.

Although the $Rh_0(D)$ zygosity of the father was evident from the isotopic data, a quantitative one to one correspondence between antibody binding and $Rh_0(D)$ zygosity was not always obtained. One R^1r child (S-5) in the Pe family took up 85% of the anti-$Rh_0(D)$ taken up by the father's homozygous R^1R^1 cells, whereas another R^1r child (D-1) took up only 56% of the anti-$Rh_0(D)$ bound to the father's R^1R^1 cells. In the Di family, an R^1r child (D-1) took up 20% more than her R^1r father. She should be genetically identical to her father, since she received his R^1 chromosome. She differs from her father only in the $Rh_0(D)$–negative chromosome, r, which she received from her mother. These results suggest, if they are not attributable to factors such as age differences between children and father, that the r (cde) chromosome is not an amorph but may influence the expression of the $Rh_0(D)$ antigenic determinant. Boettcher (42) has recently proposed a scheme for the genetic control of the Rh antigens in which the r (cde) gene is not considered an amorph with respect to the $Rh_0(D)$ antigen.

The previous studies (2–5) that have attempted to relate $Rh_0(D)$ zygosity to the quantity of cell–bound labeled anti-$Rh_0(D)$ have used unrelated individuals and have not had the advantage of the genetic resolution that is possible when the red cells from members of selected families are tested. Rochna and Hughes-Jones (5) studied unrelated individuals and had no way of unequivocally ascertaining the $Rh_0(D)$ zygosity of the individuals included in their different Rh phenotype classes. For example, the wide range of 12 to 20,000 sites reported for their R^0r subjects may be due to the presence of a homozygous R^0R^0 subject in their sample rather than to variability of the $Rh_0(D)$ antigen in this phenotype. Another important difference between their study and the present report concerns the manner in which the cell–bound anti-$Rh_0(D)$ was measured. Rochna and Hughes-Jones (5) estimated the cell–bound unlabeled anti-$Rh_0(D)$ from the uptake of ^{125}I–labeled antigamma G by the anti-$Rh_0(D)$–sensitized cells. This estimate may not be so reliable as the direct determination of the cell–bound anti-$Rh_0(D)$ used in the present study because of the uncertainties involved in determining the combining ratio of antigamma G with gamma G–anti-$Rh_0(D)$.

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