The Physiological Role of the Lymphoid System. V. The Binding of Autologous (Erythrophilic) γ-Globulin to Human Red Blood Cells*

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ABSTRACT: Human red cells and cell membranes bind certain erythrophilic components of autologous γ-globulin in considerable amounts. This is readily demonstrable *in vitro* in isotonic low ionic strength sucrose medium.

Similarly, erythrocyte-bound γ-globulin exists *in situ* in whole blood. It is reasonable to assume that the circulating red cell is coated with the erythrophilic γ-globulin. The components of erythrophilic γ-globulin correspond primarily to fractions III and IV of serum γ-globulin. Other minor components, including all isohemagglutinin activity of serum, also bind to erythrocytes under similar conditions.

In an earlier communication it was shown that dog red blood cells bind certain erythrophilic components of autologous serum γ-globulin. In particular fraction III as obtained by chromatographic separation on cellulose phosphate (CP)[1] columns was of special interest (Thomaidis *et al.*, 1967). This fraction is formed primarily by the spleen and its level in the serum is reduced after splenectomy. The decrease in half-life of the red blood cell observed in these animals was found to parallel the decrease in this γ-globulin fraction. A considerable improvement of the half-life followed parenteral supplementation which raised the level of fraction III to near normal values. Another fraction (IV) binds to a lesser extent but shows no such correlation with the survival of the cell (Fidalgo *et al.*, 1967). Human erythrocytes also bind autologous γ-globulin under similar conditions of low ionic strength (Harshman and Najjar, 1963). The membrane-bound γ-globulin was found to retard the outward flow of ions from the cell and to strengthen the membrane against shearing forces (Najjar *et al.*, 1967). This paper reports further studies on the characterization of human erythrophilic γ-globulin, which is shown to bind to the red cell *in vitro*[2] and *in situ* and to be composed primarily of CP fractions III and IV.

Materials and Methods

Human blood was obtained from adult volunteers of both sexes. It was collected with heparin as anticoagulent (25 mg/100 ml) or in glucose and citrate with final concentrations of glucose (1.6×10^{-2} M) and sodium citrate (1.6×10^{-2} M) (pH 7.4). The γ-globulin used in all erythrocyte binding experiments was obtained from the same donor (autologous). It was prepared by two precipitations at 0.33 ammonium sulfate saturation and fractionated as described in the accompanying paper (Thomaidis *et al.*, 1967) using cellulose phosphate columns (Selectacel, cellulose phosphate, Carl Scheicher & Schuell Co., Keene, N. H.). Samples containing 18–25 mg of γ-globulin previously dialyzed in acetate buffer (0.05 M, pH 4.8) at 4–6° were applied to a 1.5 × 12 cm column and fractionated at room temperature. Immunologic characterization was performed as usual (Crowle, 1961) with goat anti-γ-globulin A (anti-γA) and anti-γ-globulin M (anti-γM) sera, horse antihuman γ-globulin G (γG) (Hyland Laboratories, Los Angeles, Calif.), rabbit antihuman γ-globulin (Orthodiagnostic, Raritan, N. J.), and fluorescein-labeled rabbit antihuman γ-globulin (Immunology, Inc., Lombard, Ill). Hemagglutinin titers were obtained by standard technics (Boyd, 1956). Protein values were obtained either by optical density measurements at 280 mμ using an extinction of 1.12/mg of protein per ml or by the Folin–Lowry procedure (Lowry *et al.*, 1951). Paper electrophoresis was performed in Veronal buffer at pH 8.6 for 16 hr at room temperature. The sucrose solution used throughout was composed of sucrose (0.27 M), magnesium chloride (5×10^{-3} M), calcium chloride (5×10^{-4} M), and sodium phosphate buffer (5×10^{-3} M) at pH 7.4.

Results

Erythrocyte-Bound γ-Globulin. Human erythrocytes,

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[1] Abbreviations used: γA, γ-globulin A; γM, γ-globulin M; γG, γ-globulin G; CP, cellulose phosphate.

[2] *In vitro* refers to the binding of γ-globulin to isolated washed red blood cells. *In situ* refers to the finding of bound γ-globulin on red blood cells in freshly drawn blood with no prior manipulation of the red blood cells.

like dog erythrocytes, retain bound autologous γ-globulin when washed with the low ionic strength sucrose solution. For convenience such cells will be referred to as *coated* cells. The γ-globulin coat can be eluted readily by isotonic 0.15 M NaCl and such cells are accordingly termed *naked* cells. When *naked* cells are washed free of contaminating NaCl with sucrose solution and added to an appropriate dilution of serum in isotonic sucrose solution, only γ-globulin of all the protein components of the serum is bound to the cells in considerable quantity. However, not all the γ-globulin binds to cells even if the latter were used in large excess. This is readily apparent by contrasting the pattern of serum protein on paper electrophoresis before and after treatment with *naked* erythrocytes. Figure 1 shows the marked reduction of the γ-globulin region in the stained paper strip with the corresponding tracing of the intensity pattern. No other serum protein appears to be altered to a marked extent. Whole γ-globulin under the same conditions also shows significant binding by *naked* cells. Similarly, not all the γ-globulin was adsorbed. Chromatographic separation of the γ-globulin isolated from the supernatant of this type of experiment shows a marked reduction of fractions III and IV as compared to the pattern obtained from the γ-globulin control. This is illustrated in Figure 2. The figure also shows the fractionation pattern of the γ-globulin that was eluted from the erythrocytes with 0.15 M NaCl. It is apparent that the recovered fractions (III and IV, 55 and 35%, respectively) are those that were diminished in the supernatant. Furthermore, the red cell eluate shows a minor component (10%) corresponding to fraction II. This includes all of the γM isohemagglutinins present in that fraction. The relative amount of the latter with respect to the other two fractions is similar to that observed in the dog (Fidalgo *et al.*, 1967). In addition to the measurable reduction of these fractions caused by adsorption to the autologous red cell, all isohemagglutinin activity of the serum, which is distributed among all the four fractions, was lost from the supernatant and recoverable in the eluate (Harshman and Najjar, 1963).

The major γ-globulin fractions (III and IV) that were adsorbed on the erythrocyte in this type of experiment can also be shown to be bound to it *in situ*. Whole blood was centrifuged at 250 g and the plasma and the leucocyte layer were removed separately. The red cells were then resuspended in the clear plasma and again sedimented, followed by further removal of the contaminating leucocytes. This was repeated two or more times until most of the leucocytes were separated. The packed purified erythrocytes, still contaminating with plasma, were subjected to three successive washings each with three volumes of 0.15 M NaCl. The combined washings contained the contaminating plasma proteins and any protein that is bound to the cell membrane. The γ-globulin was then concentrated by surface evaportion in thin dialysis bags in the cold room. It was finally precipitated by adding solid ammonium sulfate to 0.33 saturation, dialyzed thoroughly against 0.05 M acetate buffer (pH 4.8), and fractionated on a

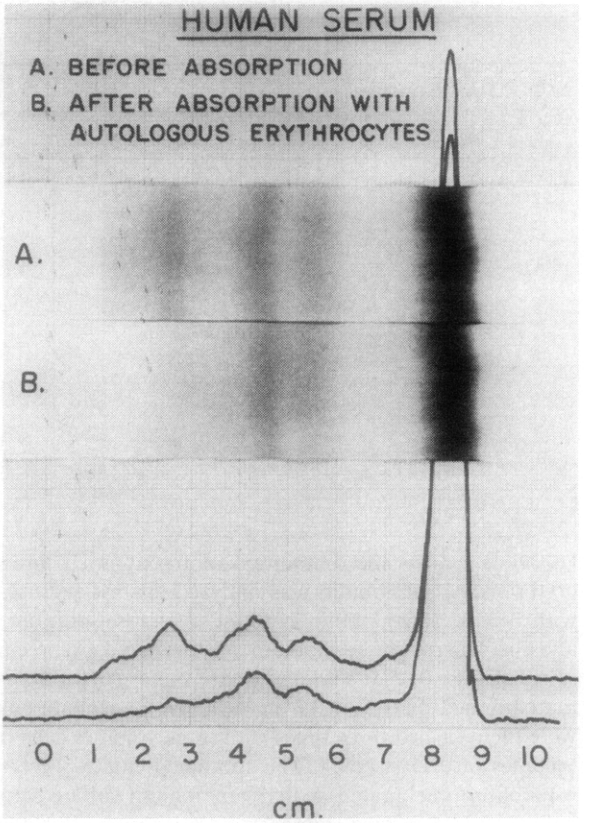

FIGURE 1: Shows the reduction of γ-globulin after adsorption on autologous naked erythrocytes. This is apparent both in the pattern on paper electrophoresis and the corresponding tracings by the analytrol. None of the other major components of serum appears to have been diminished. Human erythrocytes (O^+) (15 ml) were washed five times each with three volumes of NaCl (0.15 M) in 5×10^{-3} M sodium phosphate (pH 7.4). This was followed by washing five times, each with three volumes of the isotonic buffered sucrose solution (see text). Autologous serum was dialyzed against the sucrose solution for 2 hr. The dialyzed serum (0.5 ml) in 5 ml of sucrose solution was then mixed with 10 ml of the packed cells at 4–6° for 10 min. The supernatant (0.06 and 0.08 ml), before and after adsorption, respectively, was applied to the paper. The difference in the volume used takes into account the dilution resulting from the added erythrocytes.

CP column (Thomaidis *et al.*, 1967). The γ-globulin prepared under these conditions would necessarily include that portion derived from the contaminating plasma along with that presumably bound to the cell membrane. Thus the fractionation pattern of the isolated γ-globulin on CP columns would reflect a specific augmentation in the level of those fractions derived from membrane-bound γ-globulin. Figure 3 reveals that a considerable increase in fractions III and IV was obtained. It was anticipated that any increase in the level of fraction II would not be demonstrable under

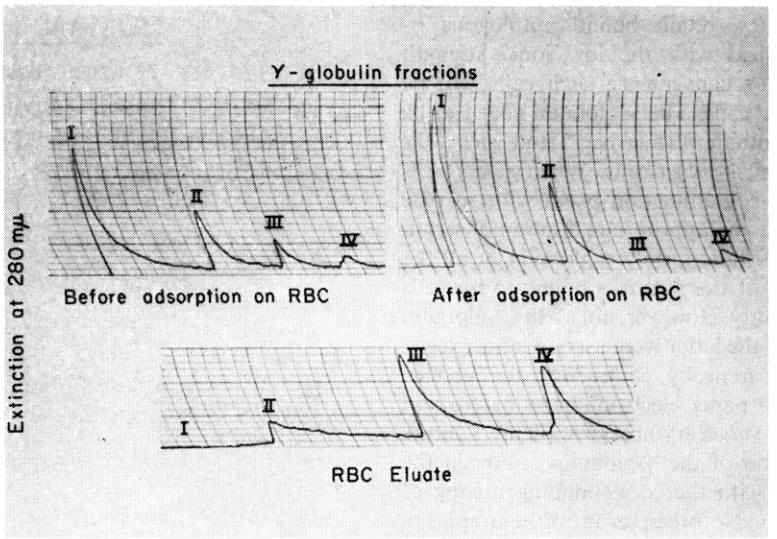

FIGURE 2: Shows the diminution of fractions III and IV after adsorbing whole γ-globulin with naked autologous erythrocytes. γ-Globulin was dialyzed against sucrose solution overnight. Packed erythrocytes (25 ml) were mixed with 100 mg of γ-globulin in 50 ml of sucrose solution. After incubation at 0° for 10 min, the sample was centrifuged and the supernatant γ-globulin was prepared by precipitation at 0.33 ammonium sulfate saturation followed by dialysis against 0.05 M sodium acetate buffer (pH 4.8). This was then chromatographed. Fifteen milligrams was chromatographed on 1.5 × 12 cm cellulose phosphate column (Thomaidis *et al.*, 1967). The cells used for adsorption were then washed three times with three volumes each of sucrose solution and finally eluted two times each with three volumes of 0.15 M NaCl. The combined eluates were evaporated in thin cellophane bags in the cold room and the γ-globulin precipitated at 0.33 ammonium sulfate saturation. The γ-globulin was prepared for chromatography as before and 15 mg was applied to the column. For comparison, unfractionated whole γ-globulin is included in the figure (upper left).

TABLE I: Agglutination of γ-Globulin-Coated Autologous Erythrocytes by Anti-γ-globulin Sera.[a]

Expt	Blood Type	State of Erythrocytes	Antiserum Used	Exposure to 0.15 M NaCl (min)	Reciprocal of Diln of Anti-γ-globulin Serum				
					4	8	16	32	64
1	O^+	Naked	Anti-γ-G	5	12	16	11	12	14
	O^+	Coated	Anti-γ-G	5	2467	1913	44	29	18
	O^+	Coated	Anti-γ-G	30	2361	2014	38	21	11
	O^+	Coated	Anti-γ-G	120	2480	1892	43	27	17
2	O^+	Naked	Anti-γA	5	15	9	7	7	6
	O^+	Coated	Anti-γA	5	2282	1961	371	18	8
	O^+	Coated	Anti-γA	30	1532	1446	488	18	9
	O^+	Coated	Anti-γA	120	1089	1740	1345	58	8
3	O^+	Naked	Anti-γM	5	9	12	12	8	8
	O^+	Coated	Anti-γM	5	1239	815	48	14	6
	O^+	Coated	Anti-γM	30	465	527	93	12	10
	O^+	Coated	Anti-γM	120	487	826	333	15	13

[a] Shows the presence of bound erythrophilic γ-globulin to red blood cells in sucrose solution. This is indicated by agglutination with rabbit anti-human γ-G. It shows further, that after reaction with antibody, the erythrophilic γ-globulin is no longer readily dissociable in 0.15 M NaCl. It is not clear why the particle counts in the experiments with anti-γA and anti-γM decreased with time at higher antiserum concentrations and increased at lower concentrations. A 0.5% red cell suspension in sucrose was prepared either with naked or coated erythrocytes. Each suspension (0.25 ml) was added to various dilutions of the antiserum in sucrose solution. At zero time, it was diluted 50-fold with 0.15 M NaCl and read in the Coulter counter at the time intervals indicated. This allowed for possible dissociation of the antibody–γ-globulin complex from the cell membrane. Coulter counter setting was 75 for maximum and 35 for minimum with a 100-μ diameter cell.

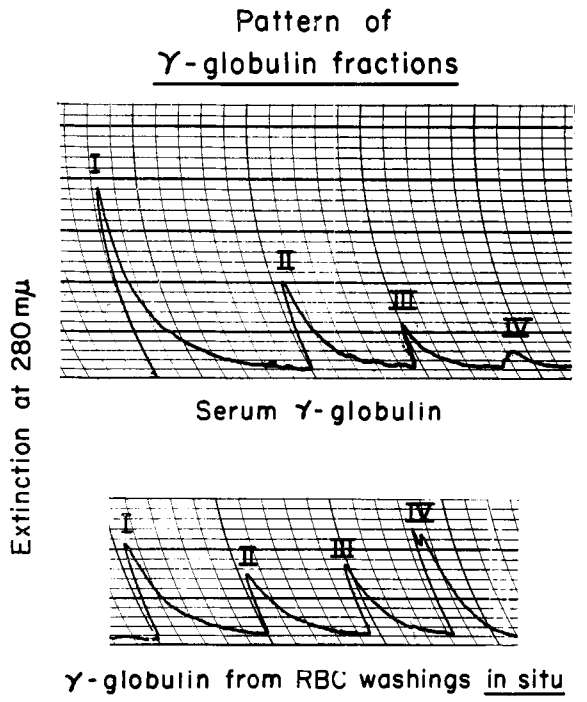

FIGURE 3: Shows the considerable augmentation of fractions III and IV in NaCl washings of erythrocytes packed *in situ*. There is also some relative increase of fraction II. This indicates that these fractions represent bound γ-globulin on the red cells under the conditions existing in blood. Blood (100 ml) containing citrate as anticoagulant was centrifuged and the clear plasma and leucocyte layer were removed. The sedimented cells were then resuspended in their plasma and recentrifuged. This was repeated three more times to minimize leucocyte contamination. Finally, the supernatant plasma was separated and the cells were washed twice with 2.5 volumes of 0.15 M NaCl each. The combined eluates (200 ml) were concentrated to a volume of 15 ml in cellophane bags by evaporation in the cold room. Solid ammonium sulfate was added to 0.33 saturation and allowed to stand for 8 hr in the cold. The precipitate was dissolved in 2 ml and dialyzed against 0.05 M sodium acetate buffer (pH 4.8) for 6 hr. It was then chromatographed on CP columns. For details, see text.

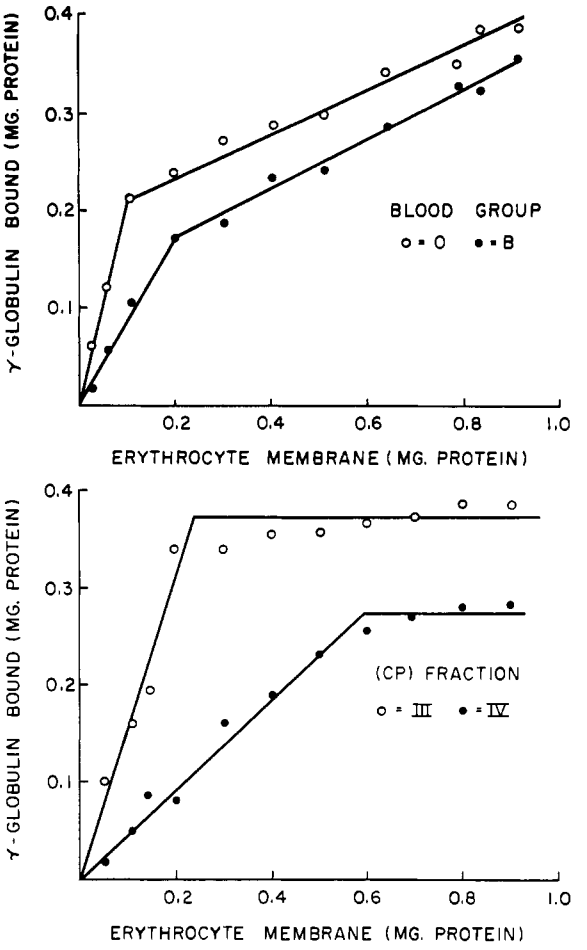

FIGURE 4: Shows that two main types of γ-globulin bind to erythrocyte membranes yielding two different slopes. The sharper slope corresponds to fraction III and the other to fraction IV. In the upper figure, γ-globulin was was isolated from eluates of red blood cells of both O^+ and B^+ types and dialyzed against sucrose solution. The γ-globulin (0.5 mg) in 1.0 ml of sucrose was pipetted into several tubes. To each tube, a variable amount of the membrane preparation was added to a final volume of 1.2 ml. These were mixed and allowed to stand at 0° for 10 min. After centrifugation, the supernatant was assayed for protein (Lowry *et al.*, 1951). In the lower figure, fractions III and IV were prepared from O^+-type blood by chromatography of γ-globulin on CP columns. The remainder of the procedure is as above. Membranes were prepared as detailed in the text.

the circumstances because of the relatively smal amount bound to the cell.

The Binding of γ-Globulin to Purified Erythrocyte Membranes. Purified erythrocytes (see above) were washed three times with five volumes each of 0.15 N NaCl and consequently freed of any bound γ-globulin and used for the preparation of cell membranes (Dodge *et al.*, 1963). The washed naked cells were hemolyzed by adding 15 volumes of hypotonic (15 milliosmolar) phosphate buffer (pH 7.4). After sedimentation at 20,000*g*, the membranes were washed four times each with seven volumes of the same buffer. The washed membranes were then repeatedly extracted with the sucrose solution in the cold room until the supernatant yielded no measurable protein after overnight extraction. Increasing quantities of these membranes were added each to separate aliquots of whole γ-globulin in sucrose solution. These were incubated with constant mixing in the cold room for 2 min and centrifuged. The amount of protein in the supernatant was then estimated. Figure 4 shows the amount of bound γ-globulin plotted against the quantity of membrane used. The figure reveals two distinct slopes (upper

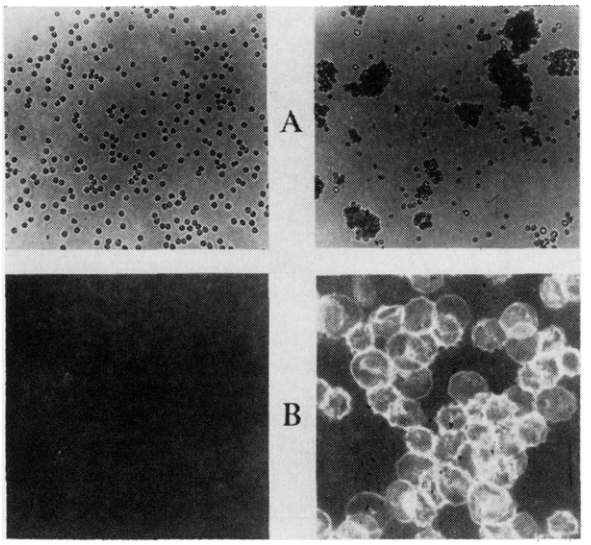

FIGURE 5: (A) Illustrates the agglutination of coated red cells carried out in sucrose solution with antihuman γ-globulin as compared to the lack of agglutination with the naked cells under identical conditions. (B) Shows the presence of fluorescence with the coated erythrocytes and the virtual absence with the naked cells. Fluorescein-labeled antihuman γ-globulin was used. The reaction was also carried out in sucrose solution. For details, see text.

figure). Similar slopes were observed in this type of experiment with whole serum γ-globulin as well as with γ-globulin eluted from coated erythrocytes. However, slight differences in the slopes with the various blood groups were observed which may be significant. The two different slopes are indicative of two separate proteins, presumably erythrophilic fractions III and IV. Consequently, a similar type of experiment was performed with the separate fractions, III and IV, isolated from serum γ-globulin by cellulose phosphate chromatography (Thomaidis *et al.*, 1967). The results are also shown in Figure 4 (lower figure) and clearly assign the steeper slope to fraction III and the other to fraction IV.

Immunochemical Demonstration of γ-Globulin Binding to Erythrocytes. Further evidence that γ-globulin binds to the erythrocyte membrane was obtained through the use of rabbit antibody against human γ-globulin. Rabbit antihuman, γG, anti-γA, and anti-γM were used as the indicator reagents. These reagents would detect the presence of γ-globulin on the membrane by causing the agglutination of coated erythrocytes. Coated erythrocytes were prepared by washing with isotonic sucrose solution, and naked cells by washing with 0.15 M NaCl as described above. The reaction was carried out in sucrose solution where γ-globulin binding to the cell membrane is maintained. After incubation with the rabbit antiserum for 1 hr at room temperature, the samples were centrifuged to accelerate possible agglutination. Figure 5A illustrates a microscopic view of the type of agglutination obtained.

It was of particular interest to determine whether the membrane-bound γ-globulin is still dissociable by isotonic salt after reaction with the rabbit antibody. To this end, the agglutinated cells were exposed to high ionic strength for variable periods. The samples were diluted 50-fold in 0.15 M NaCl and read in the Coulter counter 5 min, 30 min, and 2 hr after exposure

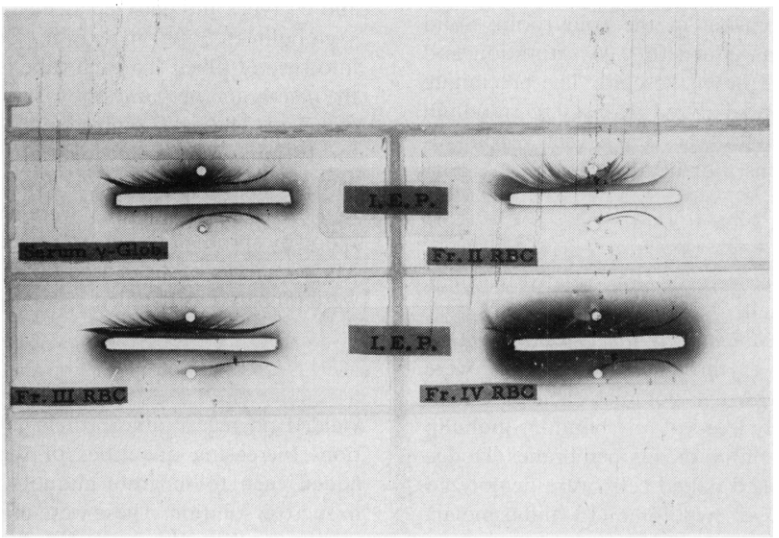

FIGURE 6: Shows that γ-globulin eluted from coated red cells (RBC) with 0.15 M NaCl contain γG as the main component and γM as the minor component of fraction II (upper). Fractions III and IV obtained from coated erythrocytes show only γG. Immunoelectrophoresis was performed on 0.10 mg of protein from the erythrocyte eluate. IEP horse antihuman serum was placed in the trough. For details, see text.

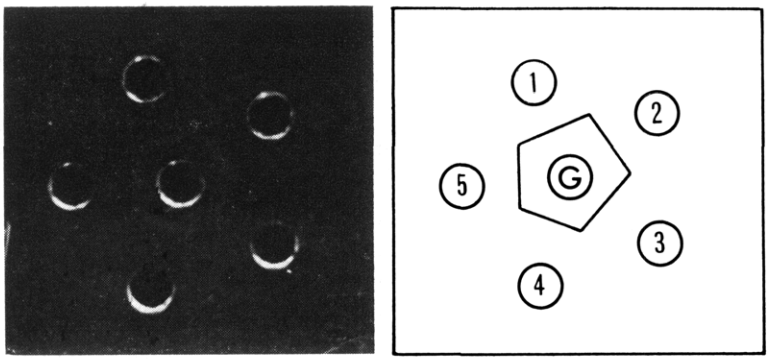

FIGURE 7: Shows that fractions III and IV from serum γ-globulin and from erythrocyte eluates have immunochemical characteristics of identity. Rabbit antihuman γG was placed in the center well (G). Ten microliters containing 50 μg of each fraction was placed in the peripheral wells and allowed to diffuse in a moist closed chamber at 4° for 48 hr. Fraction IV from serum γ-globulin was placed in 1, fraction IV from RBC eluate in 2, fraction III from serum γ-globulin in 3, and fraction III from RBC eluate in 4 and 5.

to the salt solution. The results presented in Table I show the persistence of strong agglutination which was not abolished after prolonged exposure to a high ionic concentration. It is clear therefore that following interaction with rabbit antibody the autologous γ-globulin becomes much more tightly bound to the membrane and is no longer readily dissociable. The erythrocyte-bound γ-globulin presumably undergoes considerable alteration in its tertiary structure following reaction with rabbit antibody, much as has been shown with other antigens (Najjar, 1963; Pollock, 1964; Crumpton, 1966). This results in distortion of its binding site to the membrane. A subcomplementary fit develops with interlocking of the participating groups. This in addition to the resulting hydrophobic interaction, renders the binding virtually irreversible. This interpretation is based on the analogous behavior of the two major types of antibody obtained to a single determinant, the complementary and the subcomplementary (Najjar, 1963; Najjar and Griffith, 1964).

The same reaction was carried out with the fluorescein-labeled rabbit antibody reagent. The cells were smeared on a glass slide, dried, fixed with cold absolute methanol, and incubated in a closed moist chamber at 37° for 30 min with various dilutions of the fluorescent antibody reagent. This was then washed twice with 0.15 M NaCl in 5×10^{-3} M phosphate buffer (pH 7.0), dried, and mounted for observation under dark-field illumination. Naked erythrocytes lacking a γ-globulin coat were used as control. A microscopic view of such a preparation showing fluorescence of the coated cells and the virtual lack of it with the naked cell control, is shown in Figure 5B.

The Immunochemical Characteristics of Erythrophilic γ-Globulin. Immunoelectrophoresis was performed in the usual manner (Crowle, 1961) with CP fractions II–IV derived from eluates of coated erythrocytes. Specific antisera for whole human serum and human γ-globulin were used to identify the components. Figure 6 shows that fraction II of coated cells eluates contained primarily γG and a minor γM component. On the other hand, fractions III and IV isolated from the same eluates showed only the γG component. For the purpose of comparison whole γ-globulin is included in the figure and shows the usual γG, γA, and γM components. Whole serum was used as control.

Erythrophilic fractions III and IV isolated from coated red cells have many characteristics in common with similar CP fractions isolated from serum γ-globulin: chromatographic properties, binding capacity to erythrocytes, and immunochemical characteristics of γG. Immunodiffusion (Crowle, 1961) studies shown in Figure 7 further emphasize the characteristics of immunochemical identity. Fraction III of serum γ-globulin shows immunochemical characteristics of identity with fractions III and IV obtained from the erythrocyte. Similarly, fraction IV of serum γ-globulin shows the same coalescense of the adjoining ends of its bands with both erythrophilic fractions. It can be concluded, therefore, that the erythrophilic γ-globulin that occurs *in situ* must derive from the serum γ-globulin.

The Adsorption of Isohemagglutinins by Autologous Cells. On the premise that the presence of isohemagglutinins in normal individuals represents an expression of physiological intent (Najjar, 1963), it was of interest to determine whether these also bind to the autologous red cells at conditions of low ionic strength. Accordingly, studies were carried out on whole serum, whole γ-globulin, as well as the separate fractions III and IV eluted from the cell. In an earlier communication (Harshman and Najjar, 1963), it was shown that γ-globulin bound to the red cell possesses considerable agglutinin activity with the reciprocal cell. Table II depicts representative data that extend this observation. It is apparent that exhaustive absorption of serum, serum γ-globulin, and the individual fractions thereof with the autologous cells depletes completely the isohemagglutinin activity of all the samples. This was found to be the case with blood groups A, B, and O.

TABLE II: Adsorption of Isohemagglutinins by Autologous Red Blood Cells.[a]

Sample	Test	Method	Reciprocal of Serum Dilution 32	64	128	256	512	1024
A^+ Serum								
Unadsorbed	B^+ cells	Visual	4+	+	+	+	+	−
		CPC	1187	224	47	33	30	21
Adsorbed	B^+ cells	Visual	−	−				
		CPC	21	20				
			Final γ-Globulin Concn (mg/ml) 0.250	0.125	0.063	0.031	0.016	0.008
A^+ Fraction I								
Unadsorbed	B^+ cells	Visual	2+	+	+	+	+	−
		CPC	480	97	58	34	26	16
Adsorbed	B^+ cells	Visual	−	−				
		CPC	18	12				
A^+ Fraction II								
Unadsorbed	B^+ cells	Visual	2+	+	+	+	−	−
		CPC	262	71	40	22	19	13
Adsorbed	B^+ cells	Visual	±	−				
		CPC	22	13				
A^+ Fraction III								
Unadsorbed	B^+ cells	Visual	2+	+	+	+	+	−
		CPC	308	78	42	27	25	17
Adsorbed	B^+ cells	Visual	−	−				
		CPC	19	17				
A^+ Fraction IV								
Unadsorbed	B^+ cells	Visual	2+	+	+	+	−	−
		CPC	260	66	34	26	19	13
Adsorbed	B^+ cells	Visual	−	−				
A^+ Whole γ-globulin		CPC	18	14				
Unadsorbed	B^+ cells	Visual	2+	+	+	+	+	−
		CPC	640	122	74	41	26	16
Adsorbed	B^+ cells	Visual	±	−				
		CPC	21	15				

[a] Shows that in sucrose solution, all isohemagglutinins are adsorbed by autologous erythrocytes from serum as well as from γ-globulin and its individual fractions. Serum (1 ml) was diluted with 15 ml of the buffered sucrose solution and mixed with 0.4 ml of packed autologous naked red cells. After 10 min at 0°, the sample was centrifuged and the supernatant serum as well as serial dilutions thereof were tested for agglutination against the reciprocal cell type. Aliquots of γ-globulin and γ-globulin fractions that were dialyzed in sucrose solutions were similarly treated. Erythrocytes (1 ml) was used per milligram of γ-globulin in a final volume of 3 ml. Unadsorbed serum and the various fractions were tested concurrently. Titrations with the reciprocal erythrocyte were done as usual using 0.25 ml of 0.5% cell suspension and 0.25 ml of the serum γ-globulin dilution. CPC denotes Coutler particle counter values at settings of 75 maximum, 35 minimum, and 100-μ diameter cell.

Discussion

Recent studies designed to test the validity of the proposed physiological function of γ-globulin (Najjar, 1963) revealed that γ-globulin binds to the human red cell and affects the permeability and tensile strength of its membrane (Najjar *et al.*, 1967). The studies reported herein extend these observations and those reported in the dog to show that the human red cell binds certain fractions of serum γ-globulin. The major component quantitatively is fraction III about 55% followed by IV about 35% and fraction II about 10%. While the relative preponderance of one fraction over another need not have any direct relevance to function, the experiments on the dog (Fidalgo *et al.*, 1967) indicate that fraction III is intimately concerned not only as the major bound component but also with the viability of the red cell. For that reason it was termed erythrophilic γ-globulin III in contrast to the leucophilic fraction IV that binds predominantly to autologous

leucocytes and greatly enhances the phagocytic ability of that cell (Fidalgo and Najjar, 1967a).

It appears certain from the present study that the human erythrocyte is coated with γ-globulin *in situ* as was also shown to be the case with human leucocytes (Fidalgo and Najjar, 1967b) and dog erythrocytes (Fidalgo *et al.*, 1967). It can therefore be inferred that such is the case *in vivo*. The high combined concentration of fractions III and IV, 4–6 mg/ml in the various human sera studied, relative to their concentration in packed red cells (0.4–0.6 mg/ml) would explain the existence of binding *in situ* notwithstanding the apparent physiological ionic strength of the plasma. It has already been demonstrated that at higher ionic strength, a higher concentration of leucophilic fraction IV is needed to bind to the leucocytes in order to effect a maximal phagocytic rate (Fidalgo and Najjar, 1967a,b).

Acknowledgment

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