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These findings emphasize the need to consider differences in antigen site density when comparing blood group systems. They are consistent with the hypothesis that those blood group antigens which have a very low site number will not be detected by IgG antibodies in saline hemagglutination determinations.

The Significance of Erythrocyte Antigen Site Density

I. HEMAGGLUTINATION

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ABSTRACT The importance of antigen site density has been studied by means of a model passive hemagglutination system using human red cells coupled with sulfanilic acid groups. Relative site numbers were estimated from the covalent linkage of sulfanilic acid-^{35}S to red cell membrane protein and the effective antigen site number was determined with ^{125}I-labeled rabbit IgG anti–sulfanilic acid.

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INTRODUCTION

Erythrocyte antigens and their specific antibodies may be detected and quantified by immune hemagglutination. The usefulness of this method is clearly established even though some blood group systems demonstrate complex and incompletely understood patterns of activity. Much of this heterogeneity is due to the differences in agglutinating efficiencies of IgG and IgM antibodies (1, 2). Within each antibody class, however, there are examples of both saline agglutinins and sensitizing "non-agglutinating" antibodies (3). This heterogeneity of behavior has been ascribed by reviewers of this field as probably due to differences in the nature and distribution of antigen sites on the erythrocyte membrane (3, 4).

We have tested this hypothesis by means of a model hemagglutination system in which the number of antigen sites per cell could be controlled and measured. The agglutinating properties of purified rabbit IgG and IgM anti–sulfanilic acid (anti-S) antibodies were determined using human red cells (RBC) to which were coupled variable numbers of sulfanilic acid molecules. This report considers the properties of these cells, the methods by which antigen site number may be established, and the effect of antigen site density on hemagglutination.

METHODS

Antisera. The immunizing antigen was prepared by coupling diazotized sulfanilic acid with edestin by methods previously described (5). Young adult New Zealand albino rabbits were immunized by intravenous injection of 5 mg of sulfanilic acid–azo–edestin (S-Ed) three times a week for two courses of 4 wk separated by a 7 wk period. The animals were bled on the 5th and 6th days after the last injection and the sera were stored at −20°C until used.

Preparation of purified antibody. Serum dialyzed against borate-saline buffer, pH 7.85, was applied to columns of a sulfanilic acid–cellulose immunoadsorbent (5, 6). Preliminary studies using a microcolumn assay (6) demonstrated no significant elution of bound anti-S with a pH 3, 0.05 M phosphate buffer, 1 M in NaCl, or with 0.1 N HCl. The antibody was, therefore, eluted with the specific hapten using 0.1 M sulfanilic acid with 1.0 M NaCl, pH 7.3, and dialyzed against six changes of borate-saline. Antibody recovery in the eluate averaged 60% of that in the serum applied to the column (hemagglutination assays of five separate experi-

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ments indicated 45–73% recovery). IgG and IgM antibodies were separated by gel filtration through Sephadex G-200 after the eluate was concentrated by ultrafiltration. Details of the purification methods have been reported (7). The purified IgM and IgG antibodies demonstrated single lines on immunoelectrophoresis when tested with a potent sheep anti-whole rabbit serum. Immunodiffusion assays using this serum detected IgM at concentrations as low as 0.04 mg/ml and IgG at 0.005 mg/ml. As the purified rabbit antibodies were tested at 3.10 (IgG) and 2.55 (IgM) mg of protein/ml, significant (over 1%) contamination of either fraction would have been detected. Precipitating antibody was measured by a quantitative micro method (8) using bovine serum albumin-sulfanilic acid (S-BSA) (5). Fluorescein-labeled purified IgG anti-S was prepared by conjugating 3.1 mg of protein with 1.0 mg of fluorescein isothiocyanate for 1 hr at pH 8.0 and removing the unbound dye by Sephadex G-25 gel filtration.

Preparation of sulfanilic acid–azo-human erythrocytes (S-RBC). Blood collected from normal donors was anticoagulated with acid-citrate-dextrose (ACD) (vacutainers containing solution B, Becton-Dickinson & Co., Rutherford, N. J.) and used within 4 days. A single stock preparation of ^{35}S-diazotized sulfanilic acid was used for all of the experiments reported. 1.1 mM $NaNO_2$ (76 mg) in 2 ml of distilled water (at 4°C) was added to 1 mM sulfanilic acid (10.6 mg of sulfanilic acid-^{35}S, The Radiochemical Centre, Amersham, England, 20 mCi/mmole, plus 162.0 mg of nonradioactive sulfanilic acid, Eastman Kodak Co., Rochester, N. Y.) which was dissolved in 8 ml of 0.5 N HCl at 4°C. After 15 min of diazotization, 5 mg of sulfamic acid was added and the starch iodide test changed from weakly positive to negative. The pH was adjusted to 7.5 with 5 N NaOH after 6.7 ml of 0.15 M Na_2PO_4 was added. The volume was brought to 100 ml with phosphate-saline buffer, 0.05 M in phosphate, 0.1 M in NaCl, pH 7.4, and the light yellow 0.01 M solution was stored at −20°C in 2.5-ml aliquots.

Freshly thawed diazotized sulfanilic acid was diluted with cold phosphate-saline to the desired concentration and added dropwise to five volumes of three times washed 10–20% (v/v) erythrocytes in cold (4°C) phosphate-saline. The cells were slowly stirred during the 10 min coupling period and were then washed twice with 40 volumes of phosphate-saline and once with Veronal-buffered saline (VBS). The cells were used within 6 hr of preparation. Erythrocytes from a single group B donor were used for most experiments but there were insignificant differences in S-RBC prepared using group A or O RBC.

Preparation of RBC ghosts, water washed stroma, and membrane protein. 5 ml of a 25% suspension of S-RBC were lysed by the addition of 35 ml of 0.03 M phosphate-saline-ethylenediaminetetraacetate (EDTA). After 20 min incubation at room temperature the ghosts were sedimented at 7500 *g* for 10 min at 4°C. The ghosts were subsequently washed once with 35 ml of 0.06 M phosphate-saline-EDTA and three times with 35 ml of 0.03 M phosphate-saline-EDTA (9). Water-washed stroma were prepared from the ghosts by five washes with two volumes of distilled water. Membrane protein was extracted from the water washed stroma by addition of 0.5 volume of N-butanol, vigorous agitation for 30 sec, and phase separation for 15 min at 4°C. The mixture was then centrifuged at 14,500 *g* for 15 min and the aqueous phase dialyzed at 4°C against phosphate-saline.

^{35}S measurement. 0.1 ml of a 25% (v/v) suspension of S-RBC was added to a counting vial and bleached with 0.15 ml of 60% perchloric acid and 0.3 ml of 30% H_2O_2 by heating to 70–85°C for 1 hr. 5 ml of NCS (Nuclear-Chicago Corp., Des Plaines, Ill.) solubilizer was then added followed by 9 ml of toluene which contained 6 g of 2,5-diphenyloxazole (PPO)/liter. The ghost and membrane protein preparations did not require decolorization and 0.5 ml of a 2–3 mg/ml protein suspension was digested with 2 ml of NCS before the 9 ml of toluene/PPO was added. Standard solutions of 0.1 ml of 0.1 mM sulfanilic acid-^{35}S were counted in 1 ml of NCS and 9 ml of toluene/PPO. Samples were counted using a Nuclear-Chicago automatic liquid scintillation detector and at least 10,000 counts were accumulated. Quenching was determined by channels ratio and was less than 2% for the hemoglobin-free ghost and protein samples. RBC samples had between 13 and 30% quenching.

Measurement of ^{125}I-labeled antibody binding. Purified IgG anti-S and IgG fractions of anti-S sera (7) were labeled with ^{125}I (Cambridge Nuclear Corp., Cambridge, Mass.) by the iodine monochloride (ICl) method (10). The protein was dialyzed against borate-saline and stored at 4°C until used. Over 98% of the radioactivity was precipitable by 10% trichloroacetic acid (TCA) and the labeled proteins had 1.5–2.0 iodine atoms/molecule with specific activities of 90–180 μCi/mg. Unlabeled IgG anti-S was added to these preparations to bring the protein content to 2.50 mg/ml and this mixture was absorbed twice with 0.5 volume of washed normal human RBC.

S-RBC were prepared using several concentrations of sulfanilic acid, and the number of sulfanilic acid molecules per cell was determined by means of ^{35}S measurement and a particle count using a model B Coulter counter (Coulter Electronics, Hialeah, Fla.). 1 ml of a 25% suspension of S-RBC in phosphate-saline was then incubated with 0.1 ml of dilute ^{51}Cr (Squibb, E. R., & Sons, New Brunswick, N. J., 0.7–2.0 μCi/ml for 30 min at room temperature. The cells were washed three times with VBS and a 1% suspension prepared using VBS to which had been added 2% (v/v) heat-inactivated normal human AB serum. The cell concentration was measured by means of a model B Coulter counter and the ^{51}Cr radioactivity per cell was calculated.

0.5 ml of the cell suspension and 0.5 ml of ^{125}I-labeled antibody in VBS were added to a 12 × 75 mm uncoated polystyrene tube and placed on a bench rotator at 10 rpm for 1 hr at room temperature (22–24°C). The tubes were then centrifuged (850 *g* for 5 min) and the cells transferred to a new tube using 2 ml of VBS–1% AB serum. After a second wash with 2 ml VBS–1% AB serum, the cells were lysed with 1 ml of distilled water and the tubes counted using a two channel Nuclear-Chicago crystal scintillation detector. The ^{51}Cr measurements were used to determine losses due to hemolysis and handling and these were less than 5% for the lightly coupled cells and 10–30% for the maximally coupled cells. The number of ^{125}I-labeled antibody molecules per cell was calculated using a molecular weight of 140,000 for rabbit IgG (11) and assuming that each IgG molecule was attached to a single RBC site.

Agglutination titers. 0.1 ml of a dilution of anti-S serum or purified anti-S was added to 0.1 ml of a 2½% suspension of S-RBC in VBS–1% normal human serum. After incubation for 1 hr at room temperature, the 10 × 60 mm glass tubes were centrifuged (200 *g* for 1 min) and the agglutination pattern read macroscopically. The degree of agglutination was graded from + to 4+ and the end point was recorded as the reciprocal of the highest dilution giving a + pattern. Tubes in which there was no agglutination were tested for RBC sensitization after the cells were

washed three times with 2 ml of 0.9% NaCl. 0.1 ml of an optimal dilution of sheep anti-whole rabbit serum was added to the washed cells suspended in 0.1 ml of 0.9% NaCl. The agglutination pattern was read after immediate centrifugation (200 *g* for 1 min).

The agglutination titers of B cells were determined with dilutions of an anti-B serum (Dade Reagents Inc., Miami, Fla.).

Electrophoretic mobility. The electrophoretic mobility of S-RBC was measured with the chamber described by Fuhrmann and Ruhenstroth-Bauer (12). The washed RBC were suspended in a buffer prepared by mixing one part 0.067 M Sorensen's phosphate buffer with four parts 5% sorbitol, pH 7.2, and the results were corrected for the conductivity of the medium (12). Each value reported represents the average of 10 observations in both directions with successive measurements of each cell being made by reversal of polarity.

Protein measurement. The Lowry phenol method (13) was used with human Cohn Fraction II (Pentex, Inc., Kankakee, Ill.) as standard for measurement of immunoglobulin preparations and with ovalbumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) as reference standard for determinations of RBC membrane protein.

Buffer solutions. The composition of the 10% borate-saline, pH 7.85 (5), the VBS ([14] alternative procedure with calcium and magnesium omitted), and the hypotonic phosphate-saline-EDTA (9) buffers have been previously described. The isotonic phosphate-saline used in the coupling steps was prepared by combining 80 ml of 0.15 M KH_2PO_4, 420 ml of 0.15 M Na_2HPO_4, and 1000 ml of 0.15 M NaCl and adjusting the pH to 7.4.

RESULTS

The development of a useful model system for study of changes in erythrocyte antigen content required clear definition of the nature of the antigen-RBC linkage and establishment of reproducible means for quantifying the number of sulfanilic acid molecules per cell. It was also important to distinguish the number of antigen sites per cell from the number of sulfanilic acid molecules per cell because the immunologic reactivity of some hapten groups might be reduced due to steric interference or coupling to membrane other than that at the exposed cell surface. The antigen site density of S-RBC reflects the number of antigen sites per cell, and the terms have been used interchangeably in the absence of information about the distribution of these sites on the red cell surface.

Cells coupled with sulfanilic acid-^{35}S had constant radioactivity after three washes with VBS but some loosely associated hapten was eluted only as the S-RBC were washed with VBS–1% albumin (Table I). The weakly bound hapten had no apparent immunologic significance, however, and in six separate experiments the hemagglutination titers of S-RBC washed with VBS-albumin were the same as those for cells washed only with VBS. The firmly bound ^{35}S per cell was measured by determining the radioactivity of hemoglobin-free ghosts prepared from S-RBC and these values are given for all subsequent experiments. Albumin washed S-RBC had similar ^{35}S content (Table I).

TABLE I

Sulfanilic Acid-^{35}S Distribution

Sample	Sulfanilic acid molecules per particle*	Sulfanilic acid molecules per $\mu\mu g$ protein*
S-RBC‡	2,050,000	
S-RBC washed three times with VBS–1% albumin	1,660,000	
S-RBC washed six times with VBS–1% albumin	1,730,000	
S-Ghosts	1,700,000	
S-RBC§	450,000	
S-Ghosts	295,000	460,000
S-Ghosts washed twice with 0.15 M NaCl	281,000	475,000
S-Ghosts washed twice with 1.0 M NaCl	228,000	410,000
S-Ghosts washed five times with distilled water	‖	500,000
S-Membrane protein extracted with butanol		407,000

* Average of duplicate determinations of particle number, radioactivity, and protein.

‡ Cells coupled with 1.6 mM diazotized sulfanilic acid.

§ Cells coupled with 0.33 mM diazotized sulfanilic acid.

‖ Particle counts cannot be obtained for the water washed ghosts (stroma) because of fragmentation associated with washing ghosts in distilled water.

The stability of the S-membrane linkage was established by several criteria. Neither 0.15 M NaCl nor 1 M NaCl eluted radioactivity from ghosts, and direct evidence of a stable diazo bond was obtained by measuring the specific activity of butanol-extracted membrane protein (Table I). The sulfanilic acid-^{35}S was coupled to the membrane protein by a linkage which was not affected by the multiple steps of this protein separation (repeated washes with hypotonic buffers and distilled water, extraction with butanol, and dialysis). Higgins and Harrington have established that diazotized sulfanilic acid forms covalent linkages with tyrosine, histidine, and lysine when proteins are coupled under the conditions of our experiments (15). Berg's recent studies provide additional direct evidence for covalent diazo linkage of sulfanilic acid with RBC membrane protein (16).

The number of sulfanilic acid–^{35}S molecules per cell was adjusted by varying the concentration of sulfanilic acid used for coupling (Fig. 1). The number of antigen sites on the red cell membrane was then determined by measuring ^{125}I-labeled anti-S binding to the S-RBC. Purified IgG anti-S and IgG prepared from high titer anti-S sera were used and nonspecific binding of the ^{125}I-labeled globulin to normal RBC (0.02–0.3% of radioactivity added) was subtracted from the values obtained for the S-RBC. The equilibrium concentration of antigen-antibody complex (^{125}I-labeled IgG bound to S-RBC) was determined at several concentrations of free antibody for each S-RBC preparation tested. The data of one such experiment in which three S-RBC preparations were tested are given in Fig. 2. This Scatchard plot makes it possible to estimate the antigen site number (r) by extrapolating to r/[A] = 0 where [A] is the molar concentration of free antibody (17). The intercept approximates the maximum specific binding of antibody by the red cells (18).

While antibody binding measurements provide the most accurate estimation of antigen site number, it is not practical to assess the coupling of every S-RBC preparation in this way. A reproducible relationship between the antigen site number and the total number of sulfanilic acid–^{35}S molecules per cell was demonstrated, however, when data for seven different S-RBC preparations were plotted (Fig. 3). The ratio of antigen sites (the intercept of data plotted as in Fig. 2) to sulfanilic acid–^{35}S groups was between 0.10 (minimally coupled cells) and 0.15 (maximally coupled cells). The reason for the somewhat greater ratio of antigen sites to sulfanilic acid groups for more heavily coupled cells is not known, but does not affect the clearly established relationship of the two measurements indicated in Fig. 3. All of the measured values for antigen site number were within 10% of those on the "best fit" curve drawn

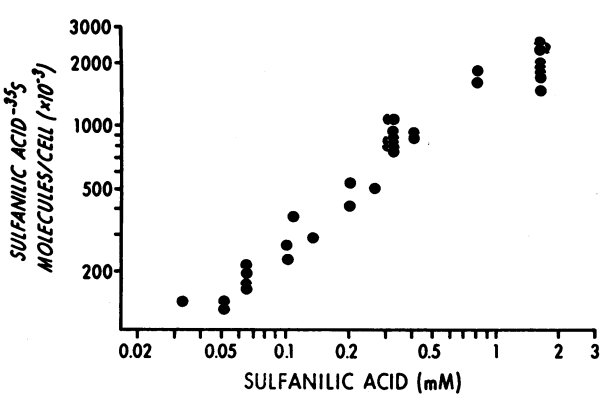

FIGURE 1 The relationship of sulfanilic acid–^{35}S molecules per cell to the concentration of sulfanilic acid in the coupling mixture. The radioactivity was determined for hemoglobin-free ghosts prepared from the S-RBC.

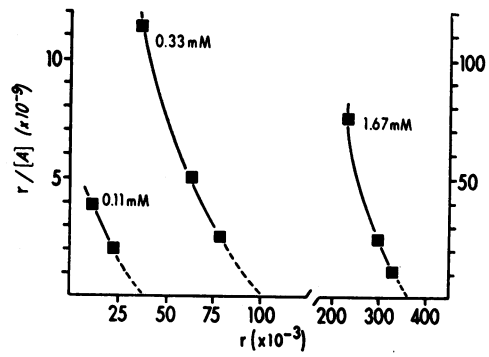

FIGURE 2 Equilibrium relationships between the number of ^{125}I-labeled IgG molecules per red cell (r) and the value r/[A], where [A] is the molar concentration of free antibody. Each point represents the average of triplicate samples. The concentrations of sulfanilic acid used in the preparation of the three S-RBC are indicated. The numbers of sulfanilic acid–^{35}S groups per ghost are (from the left) 360,000, 1,060,000, and 2,460,000. The site numbers estimated by extrapolation are 37,000, 100,000, and 360,000.

in the figure. This indicates the variability expected for values of antigen site number calculated from the graph using ^{35}S measurements.

The conditions of antibody-cell interaction were varied to be sure that the low ratio of antibody binding to sulfanilic acid molecules per cell was not due to limitations inherent in the experimental design. Equilibrium was obtained by 1 hr, and longer incubations

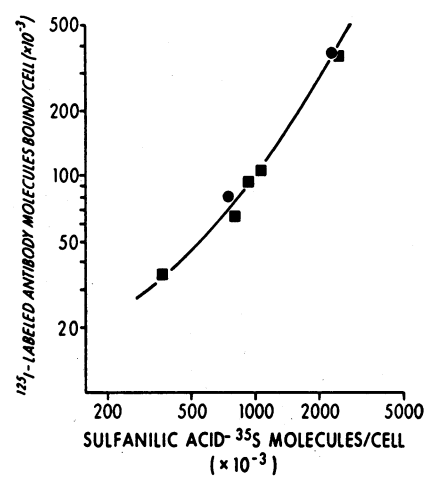

FIGURE 3 The relationship of sulfanilic acid–^{35}S molecules per cell and ^{125}I-labeled antibody binding. Each point represents the extrapolated estimate of maximal antibody binding (see Fig. 2) for a separate S-RBC preparation. The number of sulfanilic acid–^{35}S molecules per cell is determined by radioactivity measurement of ghosts prepared from the S-RBC. The values indicated by squares were obtained using ^{125}I-labeled IgG from an anti-S serum. The values indicated by circles were obtained using ^{125}I-labeled purified IgG anti-S.

did not increase the apparent site density. Incubation temperature did not affect antibody binding (37°, 25°, and 4°C were tested) and all subsequent experiments were, therefore, carried out at room temperature. Adjustment of buffer pH to 6.4 or 6.8 did not increase antibody binding, nor did reduction in the ionic strength to 0.10 or 0.06 by substitution of VBS-sucrose buffer in place of VBS (19).

The diazotized sulfanilic acid was bound to the population of red cells in a relatively uniform distribution. Agglutination patterns for cells lightly or heavily coupled with sulfanilic acid showed uniform patterns with no hint of "mixed field" reactivity. S-RBC showed uniform fluorescence after incubation with fluorescein-labeled IgG anti-S and washing with VBS (20). Although quantitative measurements of the antigenic composition of individual cells were not possible, the serologic and immunofluorescence data are consistent with uniform antigen distribution.

The relationship of antigen site number and hemagglutination was tested using S-RBC which had known antigen site densities. In addition to the ^{35}S measurements, each S-RBC preparation was tested for its agglutinating properties using a single rabbit antiserum which served as a reference throughout these studies. The combined data of 17 separate experiments are given in Fig. 4 and it is apparent that the hemagglutination measurements are consistent when cells with similar antigen site densities are compared. The agglutination titers of S-RBC prepared from each dilution of sulfanilic acid varied by no more than one doubling

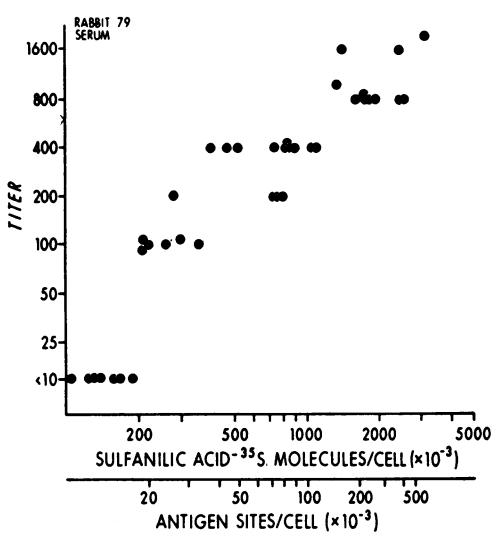

FIGURE 4 Hemagglutination titers of a single rabbit antiserum tested with S-RBC which have different antigen site densities. The abscissa indicates both the measured number of sulfanilic acid molecules per cell and the antigen site number calculated from the data of Fig. 3.

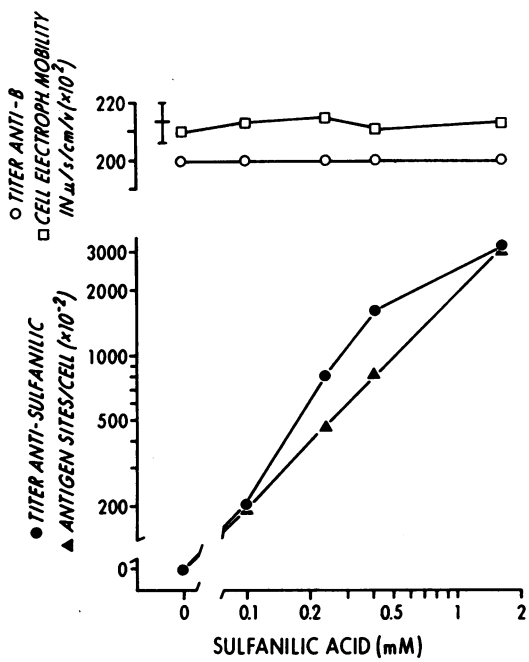

FIGURE 5 The relationship of antigen site number, specific anti-S titer, anti-B titer, and red cell electrophoretic mobility. Cells coupled with four different concentrations of sulfanilic acid are compared with normal RBC. The effective antigen site number is that calculated using ^{35}S measurements and the data of Fig. 3. The mean (±1 SD) red cell electrophoretic mobility for a group of 16 normal individuals is indicated.

dilution. The titer of the antiserum was directly related to the antigen site number above the threshold concentration of 20,000 sites per cell. No upper limit of antigen density was demonstrated above which the agglutination titer was unchanged, but cells could not be prepared with more than 400,000 antigen sites per cell.

The correlation of anti-S titer with number of sulfanilic acid groups per cell was specific for this hapten. While the anti-S agglutination titers of S-RBC prepared from B erythrocytes were directly related to the S antigen site numbers, the anti-B titers of these cells were identical (Fig. 5). Cells coupled with as many as 2,000,000 sulfanilic acid molecules per cell (300,000 antigen sites) were agglutinated by anti-B to the same titer as cells not coupled with the hapten.

As erythrocyte zeta potential very significantly affects agglutinability (21), the electrophoretic mobilities of S-RBC were compared with cells to which no hapten was coupled. The electrophoretic mobility was not affected by coupling as many as 2,000,000 sulfanilic acid molecules per cell (Fig. 5).

The hemagglutinating properties of purified IgG and IgM antibodies were determined with cells to which were coupled different numbers of sulfanilic acid groups. Cells coupled with eight different concentrations of sul-

fanilic acid-^{35}S were tested in each experiment. The purified antibody preparations had 3.10 (IgG) and 2.55 (IgM) mg of protein/ml and quantitative precipitation studies demonstrated that 72% of the protein in each preparation was precipitable with S-BSA. The data of two representative experiments are given in Fig. 6 *a* and *b*. The threshold number of antigen sites for direct agglutination was consistently higher for IgG anti-S and the maximum titer was lower. Cells with fewer than the threshold number of antigen sites did not agglutinate in the presence of 0.1 mg/ml of antibody. The greater agglutinating efficiency of IgM antibodies is evident at all antigen site densities and is 10–20 times that of IgG when samples of equal protein content are compared. Addition of an antiglobulin reagent eliminated this difference, however, and the IgG antibodies were detected in similar (Fig. 6 *a*) or slightly lower (Fig. 6 *b*) protein concentrations than IgM. S-RBC incubated with IgM were also tested with the antiglobulin reagent but in no instances were the titers increased.

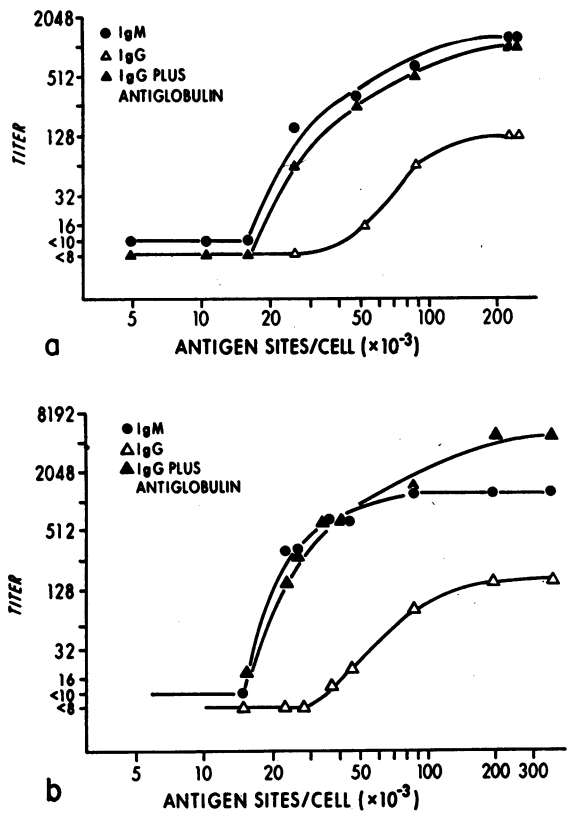

FIGURE 6 The relationship of antigen site number and the titer of purified IgM and IgG antibody. Data of two experiments are given. The titer is that calculated from dilutions of a 1 mg of protein/ml antibody preparation. The antigen site number is that calculated using ^{35}S measurements and the data of Fig. 3.

The character of the titer vs. site number relationship also differed for the two immunoglobulin classes. A consistent sigmoidal curve was obtained in IgG titrations while the IgM (or IgG plus antiglobulin reagent) titers defined a curve which was entirely concave to the abscissa. These titer vs. site number relationships were similar for IgM and IgG fractions of whole antisera as were the threshold numbers of antigen sites. It is unlikely, therefore, that the properties of the purified antibody were significantly affected by the purification procedure.

DISCUSSION

The quantitative relationship of erythrocyte antigen content and hemagglutination has been studied using a model system in which diazotized sulfanilic acid-^{35}S is coupled to human RBC. The hapten-RBC linkage has been shown to be stable during repeated washes of coupled cells and is intact in membrane protein extracted from S-RBC. Berg has recently reported detailed studies of the sulfanilic acid-erythrocyte linkage and presents additional evidence that there is a stable sulfanilic acid-membrane protein linkage (16). Sulfanilic acid is not bound to nonmembrane RBC proteins (primarily hemoglobin) under the conditions used in our experiments (16). Coupling to plasma proteins adsorbed to the RBC was minimized by thorough washing of the RBC before adding the diazotized hapten. The stability of the cell–sulfanilic acid linkage after the sequence of three washes with VBS, six washes with hypotonic buffers (in which steps osmotic lysis is achieved and the membranes are suspended in a high protein medium), and two washes with 1 M NaCl (Table I) makes it very unlikely that there was significant hapten binding to absorbed plasma proteins.

These experiments extend previous studies of the importance of antigen density on immune hemagglutination (22–24). The adsorption of certain bacterial antigens by red cells allows passive hemagglutination assays of some antibacterial antisera and Neter, Bertram, Zak, Murdock, and Arbesman established that the concentration of *Escherichia coli* antigen used for adsorption affected the agglutination titer of any given antiserum (22). The quantitative studies of Lüderetz et al. indicated that at least 5000 ^{32}P-labeled lipopolysaccharide molecules (prepared from *E. coli*) adsorbed to a red cell were necessary for agglutination and that up to 100,000 molecules may bind to each red cell (23). Ingraham's initial studies with sulfanilic acid–azo–sheep RBC (24) included several estimates of site number using ^{35}S and these data are similar to ours when differences in coupling concentration and red cell species (sheep RBC have approximately one-third the volume

and surface area of human RBC [25]) are considered. The importance of the intensity of hapten coupling on agglutination and hemolysis was established in these experiments and our studies provide a systematic evaluation of the relationship of antigen density and agglutination.

Our observations emphasize the important distinction between antigen site density and number of hapten molecules coupled to the membrane surface (Fig. 3). The distances separating effective membrane antigens can then be calculated if it is assumed that the antigen sites are distributed evenly over the 163 μ^2 surface of the human erythrocyte (25). While we have no information about the fine structural distribution of the antigen on individual cells (uniform, irregular, or clustered), the serologic and immunofluorescence data demonstrate a relatively uniform distribution of the sulfanilic acid for the population of red cells. Cells with the minimum antigen-site number needed for agglutination (20,000 sites per cell) have, with the assumption of uniform distribution, an average antigen site separation of 904 A. Electron microscopic studies using ferritin-labeled antibodies will be required to test this calculation.

Electron micrographs of IgM antibody suggest that the maximum dimension of the pentameric structure is 350–400 A (26), and it is, therefore, very unlikely that an IgM molecule could bind with two separate sites on the same red cell (multiple site IgM attachment) when low antigen density red cells are tested. Antigen densities greater than 133,000/cell would be necessary for the average separation to be as low as 350 A. If the site separation allows IgM binding to the red cell at only a single membrane antigen site, multiple site IgM attachment can not be emphasized in an interpretation of the different agglutinating properties of IgM and IgG. The greater efficiency of IgM in direct agglutination is independent of site density and is evident with cells of such low antigen content that multiple site binding is extremely unlikely. The physicochemical studies of Pollack, Hager, Reckel, Toren, and Singher (21) and Pollack (27) provide a more satisfactory basis for interpreting the differences. They relate the capacity to form intercellular bonds with antibody dimensions because agglutination is possible only if the balance of forces is such that red cells approach closely enough for antibody molecules to span the intercellular gap. IgM (a 350–400 A pentamer) would be expected to be more effective in direct agglutination than IgG (47 × 250 A [28]) because of the greater dimensions. The relatively high threshold antigen site number required for agglutination by anti-S (20,000 sites per cell) suggests that multiple intercellular bonds are required to overcome the repulsive forces associated with the RBC negative charge (21). It is difficult to account for this threshold if a single cell-antibody-cell bond is sufficient. The site number is probably important, therefore, as it affects the separation of antigen sites and the formation of multiple bonds involving small areas of the membrane surface. Cell curvature imposes a limit to the distance between antibody molecules which are effective as intercellular bridges and establishes a threshold site number for agglutination.

Greenbury, Moore, and Nunn (29) have suggested that the low hemagglutinating efficiency of IgG molecules is a result of the attachment of both antigen binding sites to a single red cell. This may be important for RBC antigens of high site density for such binding is possible for determinants like the human blood group A antigen (average separation of 128 A). Our data suggest that other factors are also important, however, for the low antigen density cells we have studied demonstrate the same difference between IgG and IgM antibodies. If the antigen sites are evenly distributed over the membrane surface, the site separation in low antigen density cells is so great that IgG attachment to two antigen sites of a single cell is very unlikely.

Mäkelä, Ruoslahti, and Ehnholm (30) and Pasanen and Mäkelä (31) have recently suggested that antibodies of different classes differ in their capacity to distinguish high and low antigen density cells. This is an inference from the agglutinating properties of human A subtype–specific antibodies (30) and hapten-specific localized hemolysis in gel of sheep RBC coupled with dinitrophenyl (31). They established that anti-A_1 formed in A_2 individuals is limited to the IgM class and they obtained absorption data consistent with the suggestion that the differences in A subtypes are due to reduced density of A-specific side chains on A_2 cells. These findings led to the hypothesis that since bivalent antibodies (IgG) can only form a single bridge between cells, the antigen density should not affect the titer and hence they should not distinguish A_1 and A_2 erythrocytes. Our findings clearly demonstrate that the titer of IgG antibodies is directly related to the antigen site number and that in this sense they "distinguish" between high and low antigen density cells. While the anti-A_1 formed in A_2 individuals may be restricted in the IgM class, this is not necessarily due to a unique capacity of this immunoglobulin class to differentiate cells with different antigen densities.

The properties of cells coupled with different concentrations of dinitrophenyl antigen provided a second instance of an apparent insensitivity of IgG antibody to antigen density (31). The evidence is indirect, however, and is limited to a comparison of the numbers of direct and "enhanced" plaques in the localized hemolysis in gel

method. Standard hemolytic titers of separated IgM and IgG antibody demonstrated significant variation in titer with changes of hapten density (31). Their data also suggested that the threshold number of antigen sites necessary for demonstrating hemolysis is greater for IgG than IgM and that this difference disappears when an antiglobulin reagent is used.

While direct hemagglutination detects IgM with greater sensitivity than IgG, this difference is not an adequate explanation for the variability of agglutination properties demonstrated by the human blood group systems. Those differences not due to antibody class or content have been interpreted as probably due to differences in the nature and distribution of the antigen determinants on the red cell membrane (3, 4). An important example is the recognition that IgG anti-A agglutinates A cells suspended in saline while IgG anti-D is always a "nonagglutinating" ("incomplete") antibody requiring some manipulation of red cell charge, the dielectric constant of the medium, or high speed centrifugation to demonstratae agglutination in the absence of an antiglobulin reagent. Most IgG antibodies resemble anti-D in this respect even though they are very different from anti-D in their capacity to fix complement (3, 4). The differences in the agglutinating capacity of IgG anti-A and anti-D may be resolved in light of the data presented here because the antigens are present in very different numbers on the RBC surface. The A antigen density is high (250,000–1,000,000/cell) (29, 32) while the D antigen density is low (5000–25,000/cell) (18, 33). As the data of Fig. 6 indicate, low density antigens may be undetectable with IgG antibodies in saline agglutination tests, however great the antibody concentration. Addition of an antiglobulin reagent is necessary to detect these IgG antibodies.

It has long been recognized that hemagglutination data must be used with caution when comparing different antigen-antibody systems and serologists have designated as "dosage effect" the greater reactivity of homozygous RBC (4). Our data emphasize the relationship of higher titer with greater antigen density and indicate that titers alone are not useful guides to comparative antibody levels.

The conclusions drawn from these studies apply with certainty only to similar hapten systems; especially those in which a negatively charged antigen is directly coupled to the membrane protein. Further studies are in progress to consider whether antigen charge (or specificity) or antigen coupling (as, for example, to bovine serum albumin which is in turn coupled to the red cell) affect the pattern of hemagglutination properties of cells with different site numbers. Standard agglutination methods were used in this study in order that the findings might be compared with usual serologic data. The demonstration of a consistent threshold antigen site number for agglutination suggests that changes in cell concentration would only affect the apparent titer and that the general pattern of data would be consistent. Manipulation of cell surface properties (enzyme treatment) or suspension medium would very likely have had a significant effect, however, and it is expected that different threshold antigen site densities could be demonstrated.These studies are in progress and will supplement the data presented here. It should be stressed that the threshold site density established for this hapten system using standard agglutination techniques (20,000 sites per cell) is an arbitrary value and considerably lower site densities may support agglutination if other variables are changed (34).

An important example of the threshold number of antigen sites necessary to detect agglutination is the standard antiglobulin (Coombs) test used to detect small numbers of IgG or complement molecules on a RBC surface, and approximately 100–300 molecules/cell are required for positive reactions (4, 35). Although the dilution at which antiglobulin sera are useful in Coombs testing corresponds to their anti-IgG or anti-complement content, the greatest dilution of a single serum which agglutinates sensitized RBC has often been used as a "Coombs titer" and related to the intensity of red cell coating (36). The findings reported here emphasize that the relationship of agglutination titer to number of antigen groups on a RBC is a general phenomenon. The marked difference in threshold number of sites between IgG as an antigen immunologically bound to the membrane (to intrinsic membrane antigens or to haptens like sulfanilic acid) and sulfanilic acid as a membrane antigen is undoubtedly related to the extension of the macromolecular antigen above the RBC surface (21). This reduces the critical distance between RBC for antibody binding and, therefore, favors agglutination.

Preliminary experiments indicate that the agglutination properties of sulfanilic acid–sheep RBC are similar to S-(human)RBC in the relationship of antigen site density to titer.[1] As S-(sheep)RBC are hemolyzed in the presence of antibody and complement (24), the model system we have characterized may also be useful in considering the effect of antigen density on hemolysis, and, more specifically, the mechanism of the hemolytic event. It will also be important to consider differences in in vivo immune cell destruction of cells which have different site numbers. The differences in complement fixation and hemolysis by antibodies of blood group systems are even more complex than the agglutinating properties of these antibodies (3, 4).

[1] Hoyer, L. W., and N. Trabold. Unpublished observations.

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