Comparison of Serum Anti-Band 3 and Anti-Gal Antibody Binding to Density-Separated Human Red Blood Cells

By Martin P. Sorette, Uri Galili, and Margaret R. Clark

This study examines the quantitative relationship between two natural serum antibodies, anti-band 3 and anti-α-galactosyl (anti-Gal), in their capacity to bind to human red blood cell (RBC) populations separated on density gradients. The question was approached in two ways. First, we determined the extent of rebinding of affinity-purified human serum antibodies to RBCs that had been stripped of in situ antibody. Second, we eluted the in situ bound antibody at low pH from density-separated RBCs and determined the proportion of total eluted antibody that bound specifically to erythrocyte band 3 or to a Gal-α-(1,3)-Gal structure. Our results show that high-density human RBCs bind increased amounts of both antibodies. Anti-Gal rebinding was specific, because it was saturable and occurred in the presence of serum IgG depleted of anti-Gal. Binding assays using control natural autoantibodies directed against antigens not found on the RBC surface showed that high-density RBCs also bind increased amounts of these antibodies as compared with low-density RBCs. However, the extent of this binding is substantially lower than that of anti-band 3 and anti-Gal. Binding studies using the lectins Bandeiraea Simplicifolia (α-galactosyl specific) and Arachis Hypogaea (peanut agglutinin, β-galactosyl specific) indicated that only the α-galactosyl sites are exposed on high density RBCs, and not the β-galactosyl structure characteristic of T antigen. Antibody that is eluted at low pH from high density RBCs contains a 5.0% to 18.0% component that binds to band 3 protein, and a 9.1% to 39.0% component that recognizes the α-galactosyl structure. Together, the two antibodies appear to constitute an average of 35% (range 17.2% to 57.4%) of the in situ bound antibody from high-density human RBCs.

SINCE ITS INITIAL demonstration by Kay,1 the binding of autologous serum antibody to high-density human red blood cells (RBCs) has been documented by many different investigators. Recently this in situ bound antibody has been the focus of interest as a possible mediator for destruction of senescent RBCs at the end of their lifespan. Although the small quantity of antibody present on circulating cells has made its characterization difficult, evidence has been obtained about the specificity of this bound antibody. Studies by Kay et al have suggested that antibody eluted from higher-density populations of human RBCs can bind to purified band 3 protein, although it does not seem to bind to intact band 3 in the whole cell.24 These investigators have proposed that the binding site in intact cells is a 60,000-Kd fragment of band 3. Another antibody bound in situ to dense human RBCs binds to α-galactosyl carbohydrate, most specifically to the Gal-α-(1-3)-Gal moiety.56 Until now, investigations of the antibody characteristics have focused on defining a single specificity, and experiments to compare the relative amounts of anti-band 3 and anti-Gal that bind to dense cells have not been reported. Thus, the present study was undertaken to provide information about the amounts of these two types of antibody that are bound to high density RBCs and what proportion of the total in situ bound antibody they comprise.

MATERIALS AND METHODS

Blood collection and density separation. After obtaining informed consent as approved by the Human and Environmental Protection Committee of the University of California, San Francisco, venous blood from healthy adult volunteers was drawn into acid-citrate-dextrose (ACD) or EDTA. In the experiments indicated, short weight (nontransfusable) units of blood drawn less than 48 hours before use were obtained from a local blood bank. Leukocytes and platelets were removed by filtration of whole blood through cellulose (Sigma Chemical Corp, St Louis, MO).7 The RBCs were washed three times in BSKG (132 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L sodium phosphate, 11 mmol/L Glucose, 290 mOsm, pH 7.4). We used arabinogalactan obtained from the St Regis Paper Co, Tacoma, WA (Stractan) or Consulting Associates, Tacoma, WA (Larex-LO) to prepare discontinuous gradients to separate cells according to their buoyant density.7 Multilayer gradients were prepared from 1.0811 to 1.1154 g/mL blood density with steps as detailed in results, layered on a cushion of at least 1.1369 g/mL density. Other gradients were designed to isolate the most dense 1%, a middle fraction, and the least dense 2% to 3% of cells, and were centrifuged in a Beckman L3-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 45 minutes at 74,000g at 4°C. The separated cells were collected from gradient interfaces using a Pasteur pipet and were washed three times in BSKG. RBC counts were determined with a Coulter Counter model B (Coulter Electronics, Hialeah, FL) to quantitate the RBC density distribution.

Nonspecific elution of in situ bound antibodies. Two standard methods were evaluated for nonspecific elution of bound antibody to provide IgG-depleted RBCs for re-binding assays. Antibodies were dissociated using heat elution into phosphate-buffered saline (PBS) at 47°C, or cold acid elution with glycine-HCl, pH 3.0, followed by rapid density gradient centrifugation to remove antibody-depleted RBCs.5 Inhibition effects on surface-volume ratio and deformability were evaluated by osmotic gradient ektacytometry.10 For isolation of in situ bound IgG to be assayed for antibody specificity, antibody was eluted with glycine-HCl, pH 2.1, cells were pelleted, and the eluates immediately adjusted to pH 7.4. Eluates were concentrated by centrifugation in Centriprep-10 concentra-

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tions (Amicon, Danvers, MA) and stored frozen at −20°C until assayed.

**Purification of band 3 protein and isolation of serum anti-band 3 antibodies.** Band 3 protein was purified using previously described methods,13 and was coupled to Affi-Gel 15 (Bio Rad Laboratories, Richmond, CA) at pH 7.4. Anti-band 3 IgG was isolated as described elsewhere,13 except that IgG was isolated from plasma by ammonium sulfate precipitation and diethyl aminomethyl (DEAE) cellulose chromatography instead of from commercial pooled IgG.

**Isolation of serum anti-galactosyl antibodies and preparation of anti-Gal-depleted serum.** Anti-Gal IgG was affinity purified by passing heat-inactivated type AB or O plasma over immunoadsorbant columns of the chemically synthesized antigen structures Gal-α-1,3-Gal-β-1,4-Glc (Synsorb 90), or Gal-α-1,3-Gal-β-1,4 GlcNAc (Synsorb 115) covalently linked to crystalline silica (Chembiomed, Edmonton, Alberta, Canada). Anti-Gal–depleted autologous serum was prepared by passage over a Synsorb 115 column six times, followed by adsorption of any residual activity by incubation with glutaraldehyde–fixed rabbit RBCs.4 Serum was tested for galactosyl reactivity by adsorption on glutaraldehyde–fixed rabbit RBCs followed by the 125I Protein A binding assay described in this section.

**Isolation of control autoantibodies.** As controls for anti-Gal and anti-band 3 autoantibodies, we used autoantibodies to proteins not found on the RBC surface. These included anti-spectrin, anti-cytochrome C, and anti-Myoglobin. Serum autoantibodies to these proteins were isolated using the procedure of Guilbert et al.17 Human RBC spectrin was isolated using established methods.17 Cytochrome C and Myoglobin were obtained from Sigma. Proteins were bound to Affi-Gel 15 (Bio Rad Laboratories) following the manufacturers instructions. Before rebinding studies, the autoantibodies were concentrated on immobilized protein A. Specificity of the anti-spectrin IgG was confirmed by Western blot analysis of RBC membrane proteins separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).19

**Assay of cell-bound IgG in rebinding studies.** Cell-bound IgG in antibody rebinding studies was determined using 125I Protein A (molecular weight 42,000 Kd; ICN, Irvine, CA) in an assay based on a previously described procedure.20 Aliquots of RBCs were incubated for 15 minutes at 37°C with 125I Protein A diluted in PBS/0.5% gelatin (200 μL/tube, concentrations ranging from 10 to 200 ng/mL, specific activity >30 μCi/μg). Nonspecific binding was estimated by adding unlabeled protein A (Sigma) in 100-fold excess. After incubation, cells were washed three times and transferred to a fresh microfuge tube containing 100 μL of 10% Stractan. The cells were pelleted through this density gradient and layer and tubes were frozen in acetone-dry ice and the cell buttons cut and counted. Because of the limited amount of available antibody, studies of rebinding of the autoantibodies directed against band 3, spectrin, myoglobin, and cytochrome C were assayed at one concentration (20 ng/tube), an amount shown to be sufficient for specifically detecting up to 500 molecules IgG/cell, based on control experiments using anti-D–adsorbed RBCs. To assure that all IgG subtypes were being detected, an identical experiment was conducted using 125I Protein G (Amersham, Arlington Heights, IL).

**Alteration of cell water content with internal cation buffer/KCl and Nystatin.** We used two methods to prepare RBCs with specified water content resulting in shrunken or swollen cells. In the first approach the antibiotic Nystatin was used as previously described21 to swell or shrink RBCs of intermediate density, and swell high-density RBCs. In this study, the stock solution was prepared by dissolving 5 mg Nystatin (Sigma) in 1 mL dimethylsulfoxide. Internal cation buffer (ICB) was prepared as described (12 mmol/L NaCl, 135 mmol/L KCl, 8 mmol/L K₂HPO₄, 2 mmol/L KH₂PO₄, 27 mmol/L sucrose, 290 mOsm, pH 7.4). The swollen or shrunken cells were isolated on Stractan density gradients as previously described, except that they were centrifuged at 20°C. In the second approach, we used ICB and KCl at the same tonicity used in the Nystatin experiments. Because RBCs swell in isotonic medium after exposure to high tonicity in the presence of Nystatin, and shrink in hypertonic medium in the absence of Nystatin, using both methods allowed us to comprehensively determine the effect of cell hydration on IgG binding independent of buffer salt concentration.

**Labeling of anti-Gal IgG and rebinding studies in anti-Gal-depleted autologous serum.** We attempted to radioiodinate anti-Gal IgG for use in rebinding studies, but found that after iodination by different protocols (Chloramine T, Bolton-Hunter, and Iodo-Gen) the immunoreactivity of the labeled antibody was unstable. We then labeled the antibody with biotin, using the N-hydroxysuccinimido ester, or a long chain analog. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NEHS-LC-Biotin, Pierce Chemical Co, Rockford, IL). IgG was biotinylated according to manufacturers instructions based on previously established methods.22,23 Comparison of labeled anti-Gal to unlabeled antibody by direct agglutination assay of rabbit erythrocytes showed that loss of reactivity was minimal, usually one twofold serial dilution. A solution of the biotinylated anti-Gal in autologous heat-inactivated serum depleted of endogenous anti-Gal was prepared at a concentration of 100 μg/mL, the approximate physiologic concentration.4 Because of the addition of labeled IgG, other serum components were diluted by only 10% to 20%. Density-separated cells were resuspended in the labeled anti-Gal containing autologous serum (106 cells/mL) and were incubated for 1 hour at 37°C. Cells were then washed three times and transferred to a new microfuge tube.22 Streptavidin (Amersham) was diluted to 1 μCi/mL in PBS/0.5% Gelatin, and cells were resuspended in 100 μL of this solution and incubated for 15 minutes at 37°C. RBCs were then washed three times, transferred to a new microfuge tube, and pelleted through 10% Stractan. Frozen cell buttons were cut and counted. To obtain an estimate of the specific activity we pepsinized rabbit RBCs with dilutions of biotin-labeled anti-Gal. These cells have been shown to express numerous α-galactosyl structures on their surface. Iodinated protein A and streptavidin binding assays were performed in parallel, and a standard curve correlating streptavidin counts to molecules IgG bound was obtained. The standard curves were linear for the range studied, and estimates of the number of bound IgG molecules were made by linear regression analysis. Data presented assume a 1:1 binding ratio of protein A to anti-Gal IgG.

**Lectin binding studies.** The ability of carbohydrate-specific lectins to bind to density-separated, acid-elicuted RBCs was determined in an assay similar to that described above. Biotinylated lectins from Bandeiraea Simplicifolia 1 and Arachis Hypogaea (peanut agglutinin, PNA) (Sigma) were diluted to a final concentration of 100 μg/mL in autologous heat-inactivated serum. To avoid competition for lectin binding sites by autologous antibody or lectin association with serum components, serum was adsorbed against a series of affinity columns. Synsorb 115 and 34 were used to adsorb α-galactosyl-and β-galactosyl–reactive antibodies, and avidin–agarose and biotin-agarose (Pierce Chemical Co) were used to remove endogenous biotin and avidin, respectively. After 1 hour of incubation at 37°C, cells were washed three times and 125I Streptavidin binding was performed as described above.

**Adsorption assay for anti-Gal IgG.** To determine the amount of IgG that had anti-Gal activity in RBC eluates, a two-step assay was used. After reserving aliquots of the eluate for assay of total IgG, the eluates were incubated together with Synsorb 115 affinity adsorbant, in polyethylene microfuge tubes pretreated overnight with 1% gelatin in PBS to prevent IgG adsorption onto the walls.
The Synsorb was added at a concentration of 400 mg/mL eluate, and the suspension incubated for 1 hour at room temperature on a rotating mixer. The tubes were then centrifuged and samples of the supernatant assayed to determine the concentration of residual unbound IgG. Control samples were run in parallel using the silica substrate without carbohydrate as an adsorbant (Chromopor P, Chembiomied). The binding of eluate IgG to the silica adsorbant was subtracted from that to the α-galactosyl-specific Synsorb 115 to give the percentage of α-galactosyl-specific binding. In addition, experiments were performed using Synsorb 34, which had a Gal β 1–4 GlcNAc carbohydrate chain, to determine the degree of cross-reactivity between the carbohydrate specific immunoadsorbants. Quantitation of IgG in the eluates and in supernatants after the adsorption step was performed by solid phase radioimmunoassay in microtitre plates. To prepare the plates for the assay, they were first treated with polymerized glutaraldehyde to minimize desorption of IgG with which the plates were subsequently coated. Glutaraldehyde was polymerized by overnight incubation at pH10. This solution was checked by UV spectrophotometry to determine that the A280/A235 ratio was less than 1.0, and brought to a concentration of 2% in PBS, pH 5.0. Microtiter plates were treated with 100 μL/well for 2 hours at room temperature. After washing three times, 100 μL of human IgG at a concentration of 10 μg/mL in 0.1 mol/L Na₂CO₃ was added to the wells and incubated for 60 minutes at 37°C. Sample eluates or standards were incubated in the wells with 171I Protein A for 60 minutes at 37°C, using a method previously described. The IgG molecules in the solution interact with 171I Protein A and this interaction inhibits its binding to the solid phase IgG. After three washes, wells were cut and counted in a γ counter. Once standard inhibition curves were obtained (bound CPM sample/bound CPM zero standard), concentrations of IgG were calculated using linear regression analysis of logit-log transformed data.

Solid-phase radioimmunometric assay for anti-band 3 IgG. Anti-band 3 IgG in the eluates was quantitated by measuring the IgG binding to band 3 protein immobilized on polystyrene microtiter plates. The plates were first washed with distilled water. After addition of 100 μL of 1% glutaraldehyde in 0.1 mol/L Na₂CO₃ (pH 9.0) to each well, the plates were incubated for 1 hour at room temperature. Another distillled water wash was followed by addition of 100 μL poly-L-lysine (Sigma) at a concentration of 20 μg/mL in 0.05 mol/L NaHCO₃ and overnight incubation at 4°C. The plates were washed and 100 μL of band 3 protein, prepared as described above, was applied to each well at a concentration of 0.1 mg/mL in 0.05 mol/L NaHCO₃ and allowed to bind overnight at 4°C. Plates were then blocked for 1 hour at room temperature with 1 mL PBS/0.05% Tween 20 containing 2% normal goat serum that had been preadsorbed on avidin/agarose and a band 3 affinity column (NGS-A). Finally, the plates were washed four times with PBS/0.05% Tween 20.

For the assay, dilutions of the eluates and standards prepared from serum anti-band 3 were analyzed simultaneously. One-hundred-microliter samples were applied to the prepared plates and incubated for 2 hours at 37°C to permit binding of the antibody to the immobilized antigen. After four washes with PBS/0.05% Tween 20/2% NGS-A, biotinylated goat antihuman IgG (100 μL, 100 ng/mL in PBS/0.5% gelatin; Vector Laboratories, Burlingame, CA) was added to detect the bound human IgG. After 1 hour at room temperature, the wells were washed four times, and 125I Streptavidin was added (1 × 10⁶ cpm/well) and allowed to bind at room temperature for 1 hour. The radioactivity measured for wells containing the diluted standard of serum anti-band 3 was used to convert radioactivity of the wells containing eluates to the amounts of band 3-specific IgG in the eluates. The concentration of IgG for serum anti-band 3 had been determined by the radioimmunoassay described above.

Quantitation of protein 4.1 a and b. Because evidence for human, mouse, and rabbit RBCs indicates that the relative proportion of the a and b components of protein 4.1 is related to cell age, we looked for alterations in the 4.1 a to b ratio in density-separated populations. Membranes were prepared from density-separated RBCs by hypotonic lysis at 4°C in 40 vol 5 mmol/L Tris-HCl/1 mmol/L EDTA/1 mmol/L diisopropyl phosphorohyd, pH 8.0. Membranes were solubilized and proteins were separated by SDS-PAGE (7% acrylamide). Proteins were stained with Coomassie blue, and the bands corresponding to 4.1 a and b were cut out and the dye eluted in 1 mL 25% pyridine and quantitated spectrophotometrically.

Statistical analysis. Paired t-test, analysis of variance (ANOVA), and linear regression analysis were performed using Statview programs (Brainpower Inc, Calabasas, CA).

RESULTS

Determination of optimal conditions for antibody rebinding studies. As previously shown, the amount of in situ bound IgG, as detected by protein A binding, was significantly increased on the most dense RBCs (Fig 1A). The experiments summarized here in which the cells were separated into 11 subpopulations of different densities show that only the most dense 1% or so of the total population is highly enriched in antibody-bearing cells. Quantitation of protein 4.1 a and 4.1 b was performed in a separate set of density gradient separations on blood from seven different individuals. The 4.1 component showed an increase relative to the b component in the bottom layers (Fig 1B), consistent with enrichment in these layers for relatively older cells in accord with the findings of other investigators.

To study the antigenic specificity of the in situ bound IgG, we eluted the bound IgG either by heat (47°C) or cold acid elution (Glycine-HCl buffer, pH 3.0). We found that low pH elution removed up to 80% of the bound IgG, whereas heat elution removed only 54%. After 5 minutes of incubation at 47°C, alterations in the deformability of the cells were detected by ektacytometry at 290 mOsm (data not shown). In contrast, cold acid elution followed by rapid density gradient centrifugation could be repeated twice with minimal effects on cell deformability. To determine further whether this treatment had deleterious effects on membrane integrity, we used osmotic gradient ektacytometry to look for changes in surface/volume relationships. We found the osmotic gradient curve was comparable with control samples of noneluted cells (data not shown).

Comparison of anti-Gal and anti-band 3 binding to density-separated, IgG-depleted RBCs. Results of antibody rebinding experiments in the absence of serum are shown in Table 1. Anti-Gal and anti-band 3 bind to all fractions of density-separated, antibody-stripped RBCs. Binding in the dense fraction is significantly increased for antibodies of both specificities. We found that the sum of binding of these two antibodies to the IgG-depleted RBCs was greater than the level of IgG bound in situ. Autoantibodies directed against spectrin, myoglobin, and cytochrome C (molecules which are not expressed on the RBC surface) were also found to preferentially associate with the dense RBC.
**BINDING OF SERUM ANTIBODIES TO HUMAN RBCs**

Fig 1. (A) Distribution of bound IgG on human RBCs from three different individuals separated on density gradients. RBCs from different individuals vary in the amount of IgG bound to the most-dense cells, but a significant increase is seen in the most-dense 1% or less of cells, indicated by the bar. (B) Proportion of protein 4.1 found in the “a” component in RBC from seven individuals separated on density gradients. Analysis of variance followed by a Tukey test demonstrates that this proportion was significantly lower (*P < .05*) in the lightest cell subpopulation (*) than cells with density greater than 1.09 (†). The proportion was significantly higher in the most-dense cell population (#) than cells with density less than 1.09.

Fig 2. Alteration of RBC hydration using Nystatin. An isolated population of intermediate-density cells (A) were treated with Nystatin to yield a fraction of shrunken RBCs with density greater than 1.1148 g/mL (B) or swollen cells with a density less than 1.0829 g/mL. Gradient D shows originally high-density cells (> 1.1148 g/mL) that were swollen by treatment with Nystatin at a tonicity of 400 mOsM and returned to isotonic medium.

**Table 1. Binding of Autoantibodies of Defined Specificity to Density-Separated Human RBCs**

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>N</th>
<th>Top</th>
<th>Middle</th>
<th>Bottom</th>
<th>IgG Bottom/IgG Middle</th>
</tr>
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<tbody>
<tr>
<td>In situ IgG</td>
<td>(6)</td>
<td>13.6 (2.3)</td>
<td>22.5 (4.7)</td>
<td>91.6 (15.3)</td>
<td>6.3 (2.5)</td>
</tr>
<tr>
<td>Anti-Gal α1,3-Gal</td>
<td>(5)</td>
<td>39.8 (5.0)</td>
<td>48.0 (9.7)</td>
<td>155.2 (20.6)</td>
<td>4.2 (1.3)</td>
</tr>
<tr>
<td>Anti-band 3</td>
<td>(5)</td>
<td>27.5 (6.3)</td>
<td>38.6 (5.0)</td>
<td>113.4 (18.9)</td>
<td>2.9 (1.4)</td>
</tr>
<tr>
<td>Anti-spectrin</td>
<td>(6)</td>
<td>31.9 (1.2)</td>
<td>69.7 (6.3)</td>
<td>2.19 (0.21)</td>
<td></td>
</tr>
<tr>
<td>Anti-cytochrome C</td>
<td>(4)</td>
<td>20.4 (3.6)</td>
<td>41.5 (4.5)</td>
<td>2.26 (0.44)</td>
<td></td>
</tr>
<tr>
<td>Anti-myoglobin</td>
<td>(4)</td>
<td>22.6 (2.5)</td>
<td>32.9 (6.0)</td>
<td>1.43 (0.12)</td>
<td></td>
</tr>
</tbody>
</table>

In situ bound IgG was measured on aliquots of density-separated cells. Cold acid eluted cells were examined for their ability to rebind putative RBC-specific autoantibodies in serum-free buffer, shown in the first three items. Binding of non-RBC surface-specific autoantibodies in serum-free buffer is shown in the last three items. Binding data are expressed as molecules protein A bound per cell, (SEM). Values in the far right column indicate the relative increase in binding on dense cells, given by the ratio of IgG bound to the most dense fraction/IgG bound to middle density cells.
Table 2. Effect of Altered RBC Hydration on Binding of Autologous Serum IgG

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tonicity (mOsm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>200</td>
</tr>
<tr>
<td>IgG rebinding to intermediate-density cells</td>
<td></td>
</tr>
<tr>
<td>ICB/KCl</td>
<td>3</td>
</tr>
<tr>
<td>Nystatin</td>
<td>3</td>
</tr>
<tr>
<td>Residual IgG on high-density cells</td>
<td></td>
</tr>
<tr>
<td>ICB/KCl</td>
<td>2</td>
</tr>
<tr>
<td>Nystatin</td>
<td>3</td>
</tr>
<tr>
<td>IgG rebinding to high-density cells</td>
<td></td>
</tr>
<tr>
<td>ICB/KCl</td>
<td>2</td>
</tr>
<tr>
<td>Nystatin</td>
<td>2</td>
</tr>
</tbody>
</table>

RBCs of intermediate and high density were isolated and their hydration status altered with hypertonic and hypotonic buffer (ICB/KCl) in the absence or presence of Nystatin. We examined the ability of RBCs of intermediate density which had been shrunken or swollen by these two methods to bind autologous serum IgG. In a separate experiment, high-density cells were swollen and examined for their ability to retain in situ bound IgG or bind autologous serum IgG. Binding data are expressed as molecules protein A bound per cell (SEM).

creased their capacity to bind autologous serum IgG. When high-density cells were swollen in hypotonic buffer, they retained their in situ bound IgG. When high-density cells were treated with Nystatin in hypotonic buffer to swell them, the swollen cells were found to have less bound IgG than the original dense cell population, although hypertonic buffer alone did not elute the bound IgG. In addition, swelling high-density RBCs did not alter their ability to bind autologous serum IgG. Thus, while extreme swelling of high-density RBCs in the presence of Nystatin results in loss of cell-bound IgG, the differential binding of IgG to cells of high density cannot be attributed to cell hydration status alone.

To further demonstrate the specificity of anti-Gal binding to RBCs of high and low density, we determined the extent to which anti-Gal IgG would bind to density-separated cells in autologous serum depleted of anti-Gal. Use of a long chain biotin analog to label the anti-Gal IgG, and adsorption of serum on biotin-agarose and avidin-agarose resulted in optimal conditions to specifically detect low levels of biotin-labeled IgG binding with ¹²⁵I Streptavidin. The total number of IgG molecules on high-density RBCs detected by this assay is much smaller than that found by rebinding assay in serum-free buffer. Nevertheless, binding to high-density RBCs is significantly enhanced compared with the binding to RBCs of lower density (Fig 3).

We further investigated the carbohydrate specificity of the galactosyl binding sites by probing with biotinylated lectins that bind to α-galactosyl structures (BS-I) or β-galactosyl structures (PNA). Top, middle, and bottom fractions of IgG-eluted cells from seven type O, Rho (D)⁺, individuals were examined for their ability to bind BS-I and PNA in the presence of autologous serum depleted of antibodies that bound to α and β galactosyl structures. There was substantial variation in the overall level of lectin binding for different individuals, but every blood sample showed increased binding of BS-I to bottom fraction cells as compared with top and middle fractions (Fig 4). In contrast, no density-associated difference was found in PNA binding. This finding further supports the presence of increased specific binding sites for anti-Gal on high-density cells. The enhancement of BS-I lectin binding to bottom layer cells was less than that observed for the rebinding of anti-Gal (Fig 3). We think this result may reflect the difference in binding affinities for the lectin and serum anti-Gal.

Anti-Gal IgG binding to high-density RBCs in the presence of other serum antibodies. To investigate the specificity of anti-Gal binding, we first performed saturation binding and competitive dissociation experiments. Anti-Gal IgG bound in a saturable manner to acid-eluted, high-density RBCs in the presence of autologous serum IgG at approximately physiologic concentrations (Fig 5A). Bound biotinylated anti-Gal IgG could not be dissociated by subsequent addition of anti-Gal-depleted serum IgG, but was displaced by unlabeled anti-Gal antibody (Fig 5B). Binding of anti-Gal was not competitively inhibited in the presence of Figure 3. Rebinding of affinity-purified anti-Gal to high- and intermediate-density RBCs in anti-Gal-depleted autologous serum. A consistent increase in binding of anti-Gal to high-density cells relative to intermediate-density cells could be detected using biotinylated antibody detected with ¹²⁵I Streptavidin.
Fig 4. Binding of galactosyl-specific lectins to density-separated RBCs. Data are normalized to represent the amount of lectin bound relative to that of the middle fraction for each individual, indicated by the dotted line. Peanut agglutinin did not show consistent differences in binding throughout the fractions (H), whereas BS-I lectin showed a significant increase (* \( P < 0.05 \)) which was density dependent (H).

It could be argued that anti-Gal, being an antibody that interacts with carbohydrate, might recognize the carbohydrate residues on the Fc portion of cell-bound IgG molecules, rather than directly interacting with \( \alpha \)-galactosyl residues on the high-density cells. To investigate this possibility, Rho(D)\(^+\) RBCs were opsonized with anti-D IgG and subsequently incubated with 100 \( \mu \)g/mL anti-Gal. No increase in anti-Gal binding to cells opsonized with 200 to 2,000 molecules of anti-D was detected. Another possible explanation for anti-Gal binding to density-separated cells is that adsorption of the arabinogalactan medium on the RBC surface promoted anti-Gal binding, because the medium is known to contain \( \alpha \)-galactosyl structures. To test this possibility, RBCs were incubated in various concentrations of Stractan prepared as for density separation. After three washes, cells were incubated with anti-Gal IgG and no alteration in binding was found (data not shown).

Anti-Gal and anti-band 3 in eluted in situ bound IgG. Eluates from middle and high-density fraction cells were tested for their ability to bind to \( \alpha \)-galactosyl immunosorbant or band 3 protein immobilized on plastic microtiter wells. In middle fraction cells, anti-Gal–reactive antibody comprised 2.5% to 6.0% of total elutable IgG (data not shown). Anti-band 3 titres for middle fraction cells fell near or below the limits of sensitivity of the assay, and are not reported. High-density cells from different individuals carried a variable percentage of the total in situ IgG with anti-Gal or anti-band 3 specificity (Fig 6). Anti-Gal contributed 9.1% to 39% of total IgG; anti-band 3 contributed 5.0% to 18.4%. In half of the individuals studied (three of six), anti-Gal and anti-band 3 IgG together comprised more than 40% of low-pH–eluted antibody.

Fig 5. (A) Anti-Gal IgG binds in a saturable manner to high-density RBCs in the presence of anti-Gal–depleted serum IgG. Mean of four experiments expressed as \( ^{3} \)H counts per minute. Vertical bars indicate SEM. (B) Biotinylated anti-Gal could not be dissociated by anti-Gal–depleted serum IgG after 1 hour of incubation (open symbols), but was displaced by unlabeled anti-Gal IgG (closed symbols). Values are the mean of two experiments.

DISCUSSION

To evaluate the proposed role of bound autologous antibody in the removal of normal RBCs from the circulation, it is useful to determine how much antibody is present on a per cell basis. In addition, it is important to assess to what extent the bound IgG is composed of antibodies that recognize specific epitopes selectively exposed on populations of RBCs. We have found that the arabinogalactan density gradient method provides a means for isolating a small population of RBCs that is highly enriched in cells that carry in situ bound antibody. Analysis of the rebinding of purified anti-Gal and anti-band 3 to antibody-stripped cells showed that both these antibodies bound selectively to the most-dense population and that the binding was in comparable amounts. Further, the binding of anti-Gal appears specific as judged from its saturability, competition
by excess anti-Gal, and lack of binding inhibition by anti-band 3 or other serum IgG. Additional experiments showed that neither cell dehydration nor adsorbed carbohydrate structures on the cell surface could explain the increased binding of anti-Gal to high-density cells. Quantitative measurement of the contributions of anti-Gal and anti-band 3 to the total in situ IgG that could be eluted from high-density cells indicated that anti-Gal constituted from 9% to 39% and anti-band 3 from 5% to 18%. Together, antibodies of these two specificities comprised from 17% to 57% of the acid-elutable IgG bound in vivo to high-density human RBCs.

Certain caveats should be kept in mind when interpreting these results. In the antibody-rebinding studies, levels of rebound antibody exceeded those found in situ. This finding may be explained by our use of affinity-purified antibodies, because the isolated clones would have a higher affinity than those which were bound in situ, but may have dissociated during the washing procedures. It is possible that the preceding antibody-stripping procedure may have enhanced the capacity of the cell to bind IgG. This possibility may explain the observed binding of non-RBC-specific antibodies, which was greater on the high-density cells. The assay of eluted in situ antibody circumvents this problem. However, the cold acid elution method used here removes only about 80% of the total IgG. Thus, the assays for anti-Gal and anti-band 3 apply only to this major fraction.

The physiologic role of in situ bound antibody and its relation to RBC senescence was not addressed in this study. It has been proposed that the bound IgG represents a marker for senescent RBCs that promotes their clearance from the circulation. One problem in testing this hypothesis is the identification and isolation of the senescent cell population. Currently, the long assumed correlation between RBC density and age is being strongly questioned. A single in vivo survival study of density-separated human RBCs suggests the most dense percent or so of cells do have markedly shortened survival, but other evidence suggests that the cells so isolated may constitute only a fraction of the total senescent population. In an effort to assess the relative age of the dense cell populations in these studies, we used a putative cell age marker that may provide a better correlation with the aging of mature cells than the previ-
ously used "age-dependent" enzymes, largely influenced by reticulocyte maturation. This marker, the protein 4.1 a to 4.1 b ratio, is the only documented example of an alteration that occurs during RBC aging in humans. This observation is supported by experimental models in mice and rabbits and appears to be a conserved feature of RBC aging in many mammals. In our experiments, the ratios of the a and b components of protein 4.1 suggest that the dense-cell populations had a greater mean age than the low-density cells, but their identity as a homogeneous population of cells on the verge of removal is not documented.

With these caveats, it is clear that the most-dense percent or so of human RBCs are an exceptional population, highly enriched in cells that carry autologous IgG on their surfaces. Although the cells show a high degree of specificity in their antibody-binding characteristics, the bound antibody expresses at least two distinct specificities, which make comparably large contributions to the total. Thus, it seems unlikely that increased antibody binding to a subpopulation of RBCs can be explained on the basis of alterations in a single membrane component, as has previously been proposed. A recent study provides evidence that mild oxidative damage to RBCs results in increased specific binding of anti-band 3 autoantibody, but not anti-Gal. Our findings suggest that during RBC aging in vivo multiple mechanisms induce alterations in membrane organization, providing a variety of previously hidden sites for binding of autoantibodies.

REFERENCES

Comparison of serum anti-band 3 and anti-Gal antibody binding to density-separated human red blood cells

MP Sorette, U Galili and MR Clark