Binding of Immunoglobulin Classes to Subpopulations of Human Red Blood Cells Separated by Density-Gradient Centrifugation

By Edward M. Alderman, H. Hugh Fudenberg, and Robert E. Lovins

Human erythrocytes (RBC) from whole blood were separated according to their specific densities by centrifugation on a polyvinyl-pyrrolidine-coated colloidal silica matrix (Percoll) into four major subpopulations. By indirect immunofluorescence assay, the most dense RBC subpopulation, with specific density greater than 1.110 g/ml (3%–5% of total RBC), was positive for membrane-bound immunoglobulin; the remaining, less dense subpopulations were negative. IgG was present on 85%–95%, IgM on 28%–32%, and IgA on 15%–20% of the RBC in the most dense population. When these immunoglobulins were eluted, radiolabeled, and used in binding studies with autologous RBC fractions subjected to thermal and/or enzymatic treatment, they reacted specifically with the less dense RBC subpopulations. These results suggest that previously cryptic antigens were revealed by the activity of neuraminidase on the plasma membranes of the treated RBC.

The normal human red blood cell (RBC) has a lifespan of approximately 120 days in vivo,1,2 after which it is thought to be sequestered and phagocytosed within the confines of the reticuloendothelial system.3 The selective nature of this removal of aging cells suggests age-related changes in the configuration or composition of the RBC surface membrane. Evidence for such membrane differences includes reported decreases in sialic acid concentration,4 phospholipid and cholesterol concentrations,5 numbers of anionic sites,6 and several enzyme activities,7 but none of these differences is currently known to promote specific phagocytosis. On the other hand, one reported difference between old and young RBC membranes that may be relevant to differential phagocytosis is the presence of autologous immunoglobulin (Ig) G exclusively on the plasma membranes of older RBC populations,8 based on the well documented changes in specific density of RBC with respect to age.9,10 In this study we have investigated RBC subpopulations, obtained by isopycnic centrifugation, for the presence of membrane-bound Ig and its distribution by class, as measured by a simple indirect immunofluorescence technique, on cells of different specific density. In addition, we have studied the nature of the binding reaction by eluting the membrane-bound Ig from older RBC and investigating its reaction with younger cells after treatment with neuraminidase.

MATERIALS AND METHODS

Initial Preparation of Red Blood Cells and Sera

Whole blood was obtained from volunteer donors and screened for ABO(H), Rh(D), and Lewis blood group specificities. Blood was drawn into sterile acid-citrate-dextrose (ACD)11 and centrifuged at 500 g for 15 min at 4°C to separate the RBC. Following removal of plasma and “buffy coat” leukocytes, the remaining RBC were washed 3–5 times by centrifugation at 4°C in 10 volumes of 0.15 M Dulbecco’s calcium- and magnesium-free phosphate-buffered saline, pH 7.4 (buffer A). The washed, leukocyte-free RBC were then suspended at 20% in buffer A and stored at 4°C for various lengths of time for later use in preliminary experiments. In subsequent experiments, the cells were used within 30 min of suspension. Sera from the same donors were subjected to precipitation in 35% ammonium sulfate to yield Ig-enriched fractions and then stored at –70°C.12

Separation of RBC Subpopulations

Subpopulations of RBC were separated on the basis of age-dependent differences in density. Washed, leukocyte-depleted RBC were fractionated on discontinuous gradients of polyvinyl-pyrrolidone-coated colloidal silica matrix (Percoll; Pharmacia Fine Chemicals, Piscataway, N.J.). A stock solution of isotonic Percoll (IP) was prepared by the addition of 1 part 10X buffer A to 9 parts Percoll, and this was further diluted in buffer A to prepare several solutions with specific density values ranging from 1.060 to 1.120 g/ml. In a typical separation, 1 x 3.5 in polyallomer centrifuge tubes (Beckman Insts., Atlanta, Ga.) with a nominal volume of 38 ml were prepared as follows: 3 ml IP, was added to each tube and was carefully overlaid with 5-ml volumes of IP, IP, and IP, and IP,. Eight milliliters of sample, either undiluted whole blood or 3 parts packed RBC to 1 part buffer A, was layered onto the discontinuous gradients, and the tubes were centrifuged at 500 g for 30 min at 22°C. The tubes were then pierced with a punch, and the cells were harvested as 1.0-mI fractions in a Gilson fraction collector. The cell fractions thus obtained were washed free of IP by centrifugation 3–5 times in 10 volumes of buffer A.

Indirect Immunofluorescence (IF) Assays

The reagents used for IF assays are listed in Table 1. All antisera, including fluorescein isothiocyanate (FITC) conjugated antisera, were obtained from Cappel Laboratories (Cochranville, Pa.). Human myeloma paraproteins were isolated from the plasma

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of patients with multiple myeloma or macroglobulinemia as described previously. The specificity and purity of the serum products were verified by immunoelectrophoresis (IEP), radial immunodiffusion (RID), and SDS-polyacrylamide gel electrophoresis.

Cells from each RBC fraction were added in quadruplicate to disposable "polyvinyl Flex V" microtiter plates (Cooke Engineering Co., Alexandria, Va.), at 5 × 10⁵ RBC/well. Immunofluorescence experiments were performed throughout at 4°C. The cells were washed by centrifugation (500 g for 5 min) 3–5 times in 200 μl of buffer A, and the supernatants were removed by aspiration. One of each quadruplicate sample was prepared for testing, and the rest were used as controls. The test cells were incubated with 6 μl of the first antiserum layer (goat anti-human IgG, goat anti-human IgM, or goat anti-human IgA) in a total volume of 150 μl (1:30 dilution) for 10 min. After 3 washes in buffer A, the cells were reincubated for 10 min in 50 μl of a 1:100 dilution of FITC-conjugated sheep anti-goat IgG antiserum, washed again, and mounted in 50% glycerol in buffer A prior to examination by fluorescence microscopy. Controls for unwanted fluorescence included substitution of buffer A (conjugate control) and of nonimmune goat serum (serum control) for the first antiserum layer. Furthermore, to reduce the risk of unwanted fluorescence, the conjugate antiserum was carried out on a Leitz Orthoplan microscope fitted with Ploem interference primary and secondary filters. An Olympus automatic epi-illumination, an HBO 200-W light source, and appropriate agitation. At the end of this incubation period, the cells were washed with ice-cold buffer A, then resuspended in the same solution to a final volume of 5.0 ml prior to further testing.

Analysis of Supernatants From Enzyme-Treated Cells

The supernatants obtained immediately before and after enzymatic treatment of the RBC fractions were assayed for the presence and concentration of N-acetyl-neuraminic acid by GLC. Trimethylsilyl (TMS) derivatives were prepared according to the procedures described by Clamp et al. Quantitation was accomplished by the incorporation of a known quantity of the internal standard, inositol, and the measurement of relative peak areas. The TMS derivatives were analyzed on an SE 30 column (Supelco Inc, Bellefonte, Pa.), at a carrier gas (N₂) flow of 20 cc/min and a programmed temperature increase of 5°C/min from 100°C to 250°C.

RESULTS

Density-Dependent Separation of RBC

Fractionation of RBC by isopycnic centrifugation on discontinuous IP gradients resulted in 35–38 1.0-ml fractions (Fig. 1). Four distinct RBC subpopulations were obtained from whole blood (A–D in Fig. 2) and only three (A–C) from leukocyte-depleted RBC. Thus, the least dense RBC (subpopulation D, specific density 1.070–1.080 g/ml) apparently was lost during the preliminary leukocyte-depletion step in the RBC purification process. Indeed, methylene blue staining
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Fig. 1. Typical separation of whole blood (enriched for aged RBC) on discontinuous isotonic-isoosmotic Percoll gradients.

Of aliquots from each of the RBC fractions confirmed that subpopulation D contained 85%-95% reticulocytes, whereas subpopulations A–C, which were common to all RBC samples, contained less than 1% reticulocytes. Subpopulation A (>1.110 g/ml) represented 3%-5% of the total RBC sample, subpopulation B (1.090–1.110 g/ml) 75%-80%, and subpopulation C (1.080–1.090 g/ml) 15%-18%.

Assays for Membrane-Bound Immunoglobulin

Examination of the various RBC fractions for human Ig by IIF revealed immunoglobulins on the surface membranes of 85%-95% of the cells (Fig. 3). IgM and IgA were detected only in the most dense of the fractions, and then only on 20%-30% of the cells. The immunofluorescence pattern on the RBC surface in the case of IgM and IgA was coarsely granular (Fig. 3). Unwanted fluorescence was not observed in either conjugate or serum control. The specificity of the reaction was further confirmed by the failure of the cells in subpopulation A to fluoresce when the first serum layer was absorbed against homologous antigen.

Although the RBC in each fraction obtained were extensively washed to remove any residual Percoll, two additional control steps were added to assess the possibility of artifactual binding of Ig to Percoll rather than to the plasma membranes of the age-separated RBC. First, all antisera were extensively absorbed against Percoll that had been pelleted by ultracentrifugation (100,000 g for 2 hr at 4°C). These absorbed sera were then used to repeat the immunofluorescence assays described above. Second, RBC previously separated on Percoll gradients were incubated with the pelleted Percoll (30 min at 37°C or 4°C), then washed by centrifugation and used in immunofluorescence assays. No significant variations from the reported results were detected in either of these control situations, indicating that contamination of the RBC fractions with residual Percoll was not responsible for the detection of Ig on only the RBC with specific density greater than 1.110 g/ml.

Table 2. RBC Fractionation on Discontinuous Percoll Gradients

<table>
<thead>
<tr>
<th>Sub-population*</th>
<th>Density Range</th>
<th>Percent of Total RBC in Sample</th>
<th>Percent Reticulocytes Detected</th>
<th>Percent Ig Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>&gt;1.110</td>
<td>3–5</td>
<td>0</td>
<td>&gt;99</td>
</tr>
<tr>
<td>B</td>
<td>1.090–1.110</td>
<td>75–80</td>
<td>0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C</td>
<td>1.080–1.090</td>
<td>15–18</td>
<td>&lt;1.0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>1.070–1.080</td>
<td>1–2</td>
<td>&gt;95</td>
<td>0</td>
</tr>
</tbody>
</table>

*As indicated in Fig. 2.
Elution of Immunoglobulin From RBC

To exclude the possibility that Ig was present on all RBC but in quantities too small to be detected in the case of subpopulations B–D, samples of each RBC subpopulation obtained from Percoll separation were subjected to heat elution. The eluates obtained were concentrated 100-fold and examined for Ig content by Ouchterlony double immunodiffusion. In the case of subpopulation A, eluates were positive for IgG, IgM, and IgA, but eluates obtained from subpopulations B, C, and D were consistently negative. These results were confirmed by studies in which the heat-elution step was preceded by surface labeling with NaB\(_3\)H\(_4\). The eluate obtained from subpopulation A yielded radiolabeled IgG, IgM, and IgA (Table 3), while the eluates from populations B–D contained no detectable Ig. The elution procedure caused only minimal cell lysis. Other methods of elution, including incubation at 56°C,\(^1\) incubation in the presence of anhydrous ether,\(^2\) and acid stromal elution,\(^3\) led to the release of surface-bound immunoglobulin, but this was consistently accompanied by extensive hemolysis. The efficiency of Ig recovery, as assessed by immunofluorescence, varied somewhat; this was particularly true for IgM, whereas removal of IgG and IgA was apparently complete. The Ig recovered retained its ability to bind to the heat-stripped RBC in subpopulation A but was not bound by the cells in subpopulations B–C, either before or after heat treatment.

Enzymatic Treatment of RBC

The quantities of N-acetyl-neuraminic (sialic) acid released by VCN hydrolysis of the RBC fractions are indicated in Fig. 4. This treatment caused minimal cell lysis, as indicated by spectrophotometric monitoring of the supernatants at 590 nm for the presence of free hemoglobin. When neuraminidase-treated cells were examined by IIF for the presence of Ig, no appreciable differences were seen in the patterns of fluorescence. The cells in RBC subpopulation A still exhibited positive fluorescence staining, indicating the presence of Ig, while those in the less dense fractions did not. However, when these procedures were repeated after preincubation of each of the RBC subpopulations with the Ig eluted from the cells in subpopulation A, all fractions exhibited positive fluorescence, indicating that IgG, IgM, and IgA were bound to antigens exposed by the removal of terminal sialic acid residues from the plasma membranes of the less dense RBC subpopulations. These results were confirmed by the use of the tritiated eluates from subpopulation A, as indicated in Fig. 4. All the cell fractions exhibited positive fluorescence, and there was a direct relationship between the amount of radiolabeled Ig bound to the cell surface and the amount of free sialic acid detected in the supernatant after neuraminidase treatment.

DISCUSSION

In these studies, Ig was detected on RBC fractions with specific density greater than 1.110 g/ml, the population previously shown to represent older RBC.\(^9,10\) Younger cells, with specific density less than
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1.110 g/ml (subpopulations B–D), were completely negative for surface-bound Ig. Analysis of the distribution of membrane-bound Ig further demonstrated that IgG was the most prevalent class, found on 85%–95% of the older RBC; IgM and IgA were found on 30% and 20%, respectively. These findings are comparable to those obtained by Kay in a previous study in which autologous IgG was detected on a lower proportion (30%) of the RBC in the aged cell population, but not on younger cells, nor was there evidence of membrane-bound IgM or IgA on any cell population. The discrepancies between the two studies can be attributed to the different methods used for separation of the RBC subpopulations and for detection of membrane-bound Ig.

The density-dependent age separation procedure utilized in Kay’s study was that originally described by Murphy, namely, high-speed centrifugation of RBC samples in a fixed-angle rotor at 30°C. In our hands, this procedure resulted in less than optimal resolution of discrete RBC subpopulations, with considerable lysis of the RBC in the most dense fractions and a high degree of granulocyte and reticulocyte contamination of the less dense fractions. These findings led us to consider the possibility that more stringent procedures of isopycnic centrifugation under more favorable conditions might disclose additional RBC subpopulations with additional Ig-binding capabilities. The use of a recently introduced reagent, Percoll, fulfilled our requirements for isotonic and isoosmotic solutions. The discrete RBC subpopulations obtained were easily washed free of Percoll. In addition, the scanning immunoelectron microscopy method used by Kay for the detection of membrane-bound Ig was considered too restrictive for use in this investigation, and an indirect immunofluorescence assay was substituted, precluding the necessity for fixation and coating of RBC prior to evaluation and permitting the examination of greater numbers of viable RBC.

The Ig detected on the aged RBC subpopulation could be efficiently eluted from the cells by heating to 47°C, which produced maximal Ig release but minimal RBC membrane damage as monitored by cell lysis and hemoglobin release. In our hands, other methods of Ig elution, including 56°C elution, anhydrous ether elution, and acid elution, all led to Ig release but with unacceptable degrees of cell lysis. The eluates obtained by thermal stripping were used to confirm the reactivity of the eluted Ig with unlabeled autologous aged RBC that had been stripped of surface-bound Ig by heat elution. It is not yet clear whether Ig is bound to the aged RBC via the Fab or Fc region of the molecule; however, the Fab mode is currently favored. Our finding that desialylation of the cells in the less dense subpopulations by Vibrio cholera neuraminidase enables them to bind the Ig eluted from autologous RBC in the more dense subpopulations is consistent with the earlier hypothesis that “receptors” for autologous Ig present on aged RBC may arise as a result of the loss of terminal N-acetyl-neuraminic acid residues from the surface membranes of younger RBC, thus “unmasking” a previously cryptic antigen. The presence of such a cryptic antigen favors the concept that the binding of Ig to the surface of aged and to VCN-treated younger RBC is a specific, and therefore Fab-mediated, reaction and further suggests that these cell populations share a common antigen that is not expressed on heat-treated or untreated younger RBC subpopulations. Additional studies of the nature of the Ig binding to the putative “antigen” expressed on aged and VCN-treated younger RBC are currently underway in our laboratory.

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