Isolation and Characterization of an Age-Related Antigen Present on Senescent Human Red Blood Cells

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Autologous membrane-bound IgG was isolated from a subpopulation of human red blood cells (RBC) with specific density >1.110, by affinity chromatography of purified RBC membrane glycoprotein preparations using immobilized wheat germ agglutinin and immobilized anti-human immunoglobulin (Ig) as immunoabsorbents. The Ig-containing RBC membrane glycoprotein preparations using chaotropic agents yielded four peaks (Ia, lb, II, and III). Double immunodiffusion revealed the presence of Ig in the first three peaks (IgM in peak Ia, IgA in lb, and IgG in II) but not in peak III. Peak III was precipitated by the Ig-containing peaks (Ia, lb, and II) in immunodiffusion assays, suggesting that the antigenic membrane determinants responsible for the binding of autologous Ig to senescent human RBC were contained in this peak (III). Peaks Ia, lb, and II precipitate purified asialoglycophorin; peak III was reactive with purified autoantibodies directed against asialoglycophorin. These results suggest that an age-related antigen determinant(s) present on senescent human RBC is exposed by desialylation of the major sialoglycoprotein component of the RBC membrane.

In a previous investigation, we showed that red blood cells (RBC) from normal human donors could be divided into four distinct subpopulations according to their specific densities by isopycnic centrifugation on discontinuous gradients of polyvinylpyrrolidine-coated colloidal silica (Percoll). The RBC in subpopulation A (approximately 30% of the total RBC) with specific density >1.110 g/ml were shown to bear autologous immunoglobulin (Ig) on their surface membranes; the less dense RBC in subpopulations B–D did not. Since it is now well established that increased specific density is characteristic of aging human RBC, the presence of membrane-bound Ig on such cells lends support to the concept of an adaptive immune response for the removal of senescent cells from the circulation by a process of immune elimination. Such a mechanism would be expected to require the binding of Ig through the Fab portion of the molecule to antigenic determinants on the membrane of the senescent cell. Evidence favoring this hypothesis is drawn from our previous observations that membrane-bound Ig was eluted from subpopulation A (old) RBC by heating to 47°C and that treatment of the less dense RBC with *Vibrio cholerae* neuraminidase (VCN) apparently exposed a “cryptic” binding site recognized by these eluted antibodies. This RBC membrane receptor appears to be an age-related antigen. We describe the purification and characterization of this “senescent cell antigen” (SCA) and present evidence that it binds autologous Ig by a specific, Fab-mediated reaction.

MATERIALS AND METHODS

Isolation and Purification of Senescent Cell Antigen

Preparation of Red Blood Cell Subpopulations

Whole blood was obtained from 14 volunteer donors between the ages of 20 and 40 yr and screened for ABO(H), Rh(D), and Lewis blood group specificities. Plasma and “buffy coat” leukocytes were removed by aspiration after centrifugation at 500 g for 15 min at 4°C, and the remaining RBC were washed 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 1). The washed, leukocyte-depleted RBC were then suspended at 20°C in buffer 1, and RBC subpopulations were separated by density gradient centrifugation as described previously.

Solubilization and Affinity Chromatography Procedures

After washing by centrifugation in buffer 1, samples of each RBC subpopulation were subjected to hypotonic lysis in 5 mM sodium phosphate buffer, pH 7.4 (buffer 2), at 4°C. Stromata were pelleted by high speed centrifugation (20,000 g for 15 min) and washed with buffer 2 until free of hemoglobin. The hemoglobin-free stroma were then selectively solubilized using 0.5% Triton X-100 as described by Yu et al. After solubilization, the supernatant was harvested by ultracentrifugation at 100,000 g for 2 hr. Triton X-100 was removed by mixing this supernatant with SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, Calif.) in a batch procedure as described by Holloway.

Solubilized RBC membrane preparations were examined for Ig content by radial immunodiffusion (RID) and immunoelectrophoresis (IEP), then subjected to affinity chromatography over immobilized wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, Calif.). Membrane components binding with low affinity were removed from the column by washing with 2 column volumes of 3.0 M saline, and the membrane glycoproteins bearing glucosamine residues were specifically eluted with 0.1 M N-acetylglucos-
amine. (This procedure has been described previously by Liljas for the purification of glycoporphin A from RBC membrane preparations.) The fraction thus eluted was dialyzed against buffer 1, concentrated, and further fractionated by affinity chromatography over a column of anti-human Ig (Cappel Laboratories, Cochranville, Pa.) bound to AH-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) after gluteraldehyde activation as described by Cambiaso et al. This column was washed with 3.0 M saline prior to elution with 1.0 M ammonium thiocyanate. The eluted peak was further fractionated over Sephadex G-200 in buffer 1 containing 1.0 M ammonium thiocyanate. The resulting fractions were extensively dialyzed against buffer 1 and then examined for Ig content by RID and IEP.

**Characterization of Senescent Cell Antigen**

**Enzymatic Treatment of RBC Subpopulations**

The RBC fractions designated subpopulations A and B, obtained by Percoll separation, were subjected to sequential enzymatic hydrolysis with *Vibrio cholerae* neuraminidase (VCN) and *Escherichia coli* beta-galactosidase (ECG) obtained from Behring Diagnostics (Somerville, N.J.). After washing and resuspension in buffer 3 (buffer 1 + 2 mM MgCl₂) to a final concentration of 5 × 10⁸ cells/ml, 5.0 ml of RBC suspension was incubated with 1.0 ml of enzyme solution (or buffer 3) containing 25 U of VCN or 50 U of ECG for 60 min at 37°C in an agitation water bath. After the incubation period, samples were centrifuged (500 g for 15 min) and the supernatants were removed.

The efficiency of enzymatic hydrolysis was assessed in the supernatants using gas-liquid chromatography (GLC) to determine the concentrations of released N-acetylneuraminic (sialic) acid and enzyme solution (or buffer 3) containing 25 U of VCN or 50 U of ECG for 60 min at 37°C. In addition, the supernatants were subjected to lectin agglutination assays. The lectins employed were peanut agglutinin (for sialyl residues, as described previously) and IEP.

**Antibodies Directed Against the Senescent Cell Antigen**

In addition to Ig obtained during the purification of antigen (see Fig. 3), Ig preparations were also obtained by elution of intact aged RBC. Samples of the RBC in subpopulation A were subjected to lectin agglutination (see Blumenfeld et al.) then subjected to mild heat treatment for the elution of Ig from the surface membranes as described previously. An aliquot of the eluted IgG was digested with pepsin to produce F(ab')₂ fragments, which were then FITC-conjugated as described by Bergquist and Nilsson. Unlabeled F(ab')₂ and Fc fragments of IgG were prepared with pepsin and papain, respectively, as described elsewhere.

**Preparation of Glycoporphin Derivatives and Autoantibodies Directed Against Them**

Circulating autoantibodies to cryptic glycoporphin antigens were isolated from autologous normal human serum by affinity chromatography (Fig. 1); a single 5.0-ml aliquot of human Ig obtained by ammonium sulfate precipitation was serially fractionated over columns containing AH-Sepharose 4B with specifically modified glycoporphin A (from autologous RBC) covalently attached as immunosorbents. In each case, the glycoporphin A fraction was isolated from solubilized membrane preparations obtained from RBC in subpopulation B by affinity chromatography over immobilized WGA. The matrix was washed with 3.0 M saline, and glycoporphin A was specifically eluted using 0.1 M N-acetylgalactosamine. Unmodified glycoporphin A obtained from untreated RBC was used as the immunosorbent in column I, while asialoglycoporphin (AGS) obtained from VCN-treated RBC or by VCN treatment of purified glycoporphin A was used in column II. Asialoagalactoglycoporphin (ASAGG) obtained from VCN/ECG-treated RBC or by the same treatment of purified glycoporphin A was used as the immunosorbent in column III, as indicated in Fig. 1.

**Specificity of Membrane-Bound Ig**

RBC subpopulations were examined for the presence of membrane-bound Ig by indirect immunofluorescence (IF) assay as described previously. Aliquots from each RBC fraction were subjected to IF carried out at 4°C in microtiter plates, using a 1:30 dilution of goat anti-human IgG (Cappel Laboratories, Cochranville, Pa.) as the first antisera layer, and a 1:100 dilution of fluorescein isothiocyanate (FITC) conjugated rabbit anti-goat Ig (Cappel Laboratories), which had been previously absorbed against pooled human RBC, as the second antisera layer. Appropriate controls for unwanted fluorescence included substitution of buffer I (conjugate control) and of nonimmune goat serum (serum control) for the first antisera layer. Reaction specificity was indicated by absorption of each antisera with homologous antigen in the form of human myeloma paraprotein IgG, isolated as described previously.

**Analysis of Antigen and Antibody Fractions Obtained**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely performed to monitor the purity and homogeneity of immunochemical reagents and all RBC membrane fractions isolated by various chromatographic procedures. In experiments involving surface radiolabeling, the resulting gels were sliced into 2-mm portions, treated with Aquafol scintillation fluid (Beckman Instrument Co., Atlanta, Ga.), and examined by liquid scintillation counting. Ouchterlony double immunodiffusion (DD) was employed to monitor the specificities of reagents, RBC membrane fractions, and antibody preparations after purification.

The content of glycoprotein antigens in solubilized membrane fractions was assessed in terms of the ability of such fractions to inhibit the agglutination of RBC subpopulations by PNA. After determination of the optimal agglutinating dilution of lectin for each of the native RBC subpopulations, 25 μl of this dilution was added to wells 1-10 and 25 μl of buffer 1 to well 11, and 25 μl of the membrane fraction suspected to contain antigen or antibody activity was added to wells 1 and 12 in a given row. This fraction was serially diluted from well 1 through well 9, leaving well 10 as a positive and well 11 as a negative control for lectin agglutination (well 12 served as a control for agglutinating activity exhibited by the "inhibitor"). Twenty-five microliters of appropriate RBC suspension (2 × 10⁷ RBC/ml) was added to each of the wells in a row, and the loaded plates were incubated, centrifuged, and read as described above. Any test producing definite agglutination in well 12, containing inhibitor and RBC (but no lectin), was repeated using in wells 1 and 12, the highest "inhibitor" concentration that did not result in spontaneous agglutination of RBC.

**RESULTS**

**Enzymatic Treatment of RBC Subpopulations**

Enzymatic treatment of the RBC subpopulations led to no obvious alterations in the patterns of Ig...
fluorescence previously reported for untreated cells; subpopulation A cells (the oldest 3%) continued to exhibit positive fluorescence, indicating the presence of membrane-bound Ig, while the cells in subpopulation B did not. To assure that galactosyl and sialyl residues were indeed cleaved from enzymatically treated cells, TMS derivatives were prepared from supernatants obtained immediately prior to and following ECG and/or VCN treatment. These TMS derivatives were examined by gas-liquid chromatography for the presence of galactosyl and sialyl residues. The results of these experiments (Table I) indicate that greater quantities of N-acetylneuraminic acid were released from subpopulation B than from subpopulation A cells by VCN treatment. Heat-treated (Ig-eluted) subpopulation A cells apparently contained even fewer VCN-susceptible sialyl residues than untreated cells from the same subpopulation, whereas thermal treatment had little effect on the cells in subpopulation B. Further enzymatic treatment with ECG had little apparent effect on the VCN-mediated hydrolysis of sialyl residues from any of the cells.

ECG treatment led to the hydrolysis of similar quantities of galactosyl residues from subpopulation A and subpopulation B cells. Thermal treatment prior to such hydrolysis had little apparent effect on the cells from subpopulation B but led to the release of substantially greater quantities of galactosyl residues from the subpopulation A cells. In contrast, pretreatment with VCN had an opposite effect; i.e., no obvious alteration was seen in the concentration of galactosyl residues released from the cells in subpopulation A, while more such residues were stripped from sequentially treated subpopulation B cells (Table 2). Thus, it appears that thermal treatment of subpopulation A cells and VCN treatment of subpopulation B cells leads to the expo-

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**Table 1.** N-Acetylneuraminic Acid (NANA) Residues Released From RBC After Treatment With *Vibrio cholerae* Neuraminidase (VCN)

<table>
<thead>
<tr>
<th>RBC Subpopulation*</th>
<th>Treatment Prior to VCN Hydrolysis†</th>
<th>NANA in Supernatant After VCN Treatment (ng/10⁶ RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (old)</td>
<td>None</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Heat</td>
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</tr>
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<td></td>
<td>ECG</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>Heat, ECG</td>
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<td>17.7</td>
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<td></td>
<td>Heat</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>ECG</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>Heat, ECG</td>
<td>16.9</td>
</tr>
</tbody>
</table>

*As defined in reference 1.
†As described in text.
As defined in reference 1.

As described in text.

ABC

Subpopulation A (old)

B (younger)

LPAt

512

256

PNA

512

1024

16

1024

256

Ig(+)

GLYCOPHORIN A

1.0

0

20

50

FRACTION NUMBER

Fig. 2. Affinity chromatographic separation of Ig-containing fractions from RBC membrane glycoprotein preparations on immobilized wheat germ agglutinin. Arrow indicates start of elution buffer (0.1 M N-acetylglucosamine).

as sure of galactosyl residues that can be removed by treatment with ECG.

The efficiency of enzymatic treatment was also assessed by agglutination assays using PNA (galactosyl-specific) and LPA (sialyl-specific) lectins for the presence of residual galactosyl and sialyl moieties remaining on the surface of enzyme-treated cells (summarized in Table 3). Subpopulation A (old) red cells were not LPA-agglutinable under any circumstances, while cells from the less dense (younger) subpopulation were readily agglutinable by the sialyl-specific lectin. These results were not altered by thermal or ECG treatment of the subpopulations, but the agglutinability displayed by the less dense cells was lost following neuraminidase treatment.

None of the untreated RBC subpopulations was agglutinable with PNA, although neuraminidase treatment of the subpopulation B cells rendered them PNA-agglutinable, as shown in a previous report.17 Although thermal treatment caused no appreciable difference in the agglutinability displayed by these cells, their reactivity was lost following ECG treatment. In contrast, subpopulation A cells were rendered PNA-agglutinable only by thermal treatment; this reactivity was lost after subsequent ECG treatment but not after VCN treatment (Table 3).

Purification and Solubilization of RBC Membranes

Stroma prepared from RBC subpopulations A and B were assessed for enrichment of glucose-6-phosphate dehydrogenase activity, as a marker for the plasma membrane.5 After selective solubilization of membrane glycoproteins with Triton X-100,6 the detergent was removed with Bio-Beads SM-2;7 the criterion for complete removal was failure of the resulting supernatant to lyse 2% RBC suspensions in buffer 1. The yield of RBC membrane glycoprotein obtained after these procedures was about 225 μg/ml packed RBC. Comparative studies of the Ig content in these glycoprotein preparations indicated the presence of Ig in the preparations from subpopulation A cells, but not in those from subpopulation B. Consequently, fractionation procedures using affinity chromatography were initially performed on subpopulation A (old) cells exclusively.

Table 2. Galactose (GAL) Residues Released From RBC After Treatment With Escherichia coli Beta-Galactosidase (ECG)

<table>
<thead>
<tr>
<th>RBC Subpopulation*</th>
<th>Treatment Prior to ECG Hydrolysis†</th>
<th>GAL in Supernatant After ECG Treatment (ng/10^8 RBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (old)</td>
<td>None</td>
<td>18.6</td>
</tr>
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<td></td>
<td>Heat</td>
<td>55.3</td>
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<td></td>
<td>VCN</td>
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<td>Heat, VCN</td>
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<td>B (younger)</td>
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<td>19.4</td>
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<td></td>
<td>Heat</td>
<td>17.9</td>
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<td></td>
<td>VCN</td>
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<tr>
<td></td>
<td>Heat, VCN</td>
<td>66.4</td>
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</table>

*As defined in reference 1.
†As described in text.

Table 3. LPA- and PNA-Induced Agglutination of RBC Subpopulations

<table>
<thead>
<tr>
<th>RBC Subpopulation*</th>
<th>Treatment Prior to Agglutination Reaction†</th>
<th>LPA‡</th>
<th>PNA§</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (old)</td>
<td>None</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Heat</td>
<td>512</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>ECG</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>—</td>
<td>—</td>
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<td></td>
<td>Heat, ECG</td>
<td>—</td>
<td>—</td>
</tr>
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<td>Heat, VCN</td>
<td>1024</td>
<td>—</td>
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<td></td>
<td>ECG, VCN</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>VCN, ECG</td>
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</tr>
<tr>
<td></td>
<td>Heat, ECG, VCN</td>
<td>16</td>
<td>—</td>
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<tr>
<td></td>
<td>Heat, VCN, ECG</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B (younger)</td>
<td>None</td>
<td>512</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Heat</td>
<td>512</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>ECG</td>
<td>256</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>VCN</td>
<td>—</td>
<td>1024</td>
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<td></td>
<td>Heat, ECG</td>
<td>256</td>
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<td>Heat, VCN</td>
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<td>256</td>
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<td>VCN, ECG</td>
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<td></td>
<td>Heat, VCN, ECG</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*As defined in reference 1.
†Where more than one treatment is indicated, cells were treated in the order shown.
‡Limulus polyphemus agglutinin (sialyl-specific) diluted 1:100 in buffer 1 prior to use.
§Peanut agglutinin (beta-galactose-specific) diluted 1:100 in buffer 1 prior to use.
||Reciprocal of lectin dilution producing agglutination.
The first step of the isolation involved affinity chromatography over immobilized wheat germ agglutinin. Ig was shown to bind to this column and was coeluted with the major RBC membrane sialoglycoprotein (as demonstrated by the presence of MN blood group determinants) in the presence of 0.1 M N-acetylglucosamine (Fig. 2). After concentration by vacuum dialysis against buffer 1, the eluted fraction was applied to a second affinity column containing anti-human Ig bound to AH-Sepharose 4B. The Ig bound to this column was eluted with 1.0 M ammonium thiocyanate and further separated by chromatography on Sephadex G-200 columns. Four major peaks, designated Ia, Ib, II, and III, were obtained (Fig. 3). DID and RID revealed that IgM was present in peak Ia, IgA in peak Ib, and IgG in peak II. No Ig was detectable in peak III, which was tentatively identified as the senescent cell antigen (SCA). When glycoprotein preparations obtained from subpopulation A cells from which Ig had been stripped by heat elution were applied sequentially to the affinity columns as above, of which Ia, Ib, and II contained radiolabel. Specificity of Membrane-Bound Ig

IgG obtained from solubilized membrane fractions was examined in parallel with IgG eluted directly from subpopulation A RBC membranes and with Ig fractions obtained from normal human serum. As in previous experiments, thermal treatment of subpopulation A RBC led to apparently complete stripping of membrane-bound IgG and IgA, but only approximately 40% of the membrane-bound IgM was released by this procedure. Moreover, when cells from subpopulation B were used, minimal amounts of protein were found in the eluate and no detectable IgG. This procedure caused minimal cell disruption (<5%) as monitored by hemoglobin release. When thermal elution was preceded by tritium labeling of the RBC, a similar pattern of Ig release was observed.

The specificity of the IgG precipitated from these Ig fractions was assessed in several ways. First, the ability to bind to RBC subpopulations expressing the membrane Ig binding site was examined in readdition experiments, in which variously treated RBC were incubated with the different IgG preparations at 37°C. Binding, as assessed by IIF assay, indicated that all three IgG preparations were reactive with heat-eluted RBC from subpopulation A and also with VCN-treated cells from subpopulation B. Consistently negative results were obtained in the case of heat-eluted or membrane-solubilized IgG when galactosyl residues were removed from the cell surfaces of heat-eluted subpopulation A or VCN-treated subpopulation B cells by ECG treatment. In contrast, IgG fractions from normal human serum were shown to bind to both VCN- and VCN/ECG-treated cells. Comparable results were obtained using the same IgG fractions in a direct immunofluorescence assay after labeling with FITC. Furthermore, readdition of 3H-labeled Ig was evaluated by subjecting the RBC subpopulations under investigation to beta counting after incubation in the presence of the labeled IgG fraction. Cells were washed with buffer 1, harvested into counting vials, and after the addition of scintillation fluid (Aquaflo) were subjected to beta counting. These procedures revealed an identical pattern of binding; positive results were obtained only when free galactosyl residues were available for IgG binding. Use of FITC-labeled F(ab')2 fragments of Ig obtained by pepsin digestion produced a similar binding reaction, which
was blocked by preincubation with unlabeled F(ab')₂ but not by Fc fragments. Thus, all IgG fractions present on the RBC appeared to be bound to the relevant membrane components through the Fab portion of the IgG molecule.

The specificity of the Ig fractions was also examined in terms of their ability to block lectin-induced agglutination of heat-treated cells from subpopulation A. All three peaks (Ia, Ib, and II) inhibited the agglutination induced by PNA (Table 4).

Additional studies of Ig specificity were performed in relation to reactivity with glycophorin A isolated from subpopulation B RBC, both in the native state and after chemical modification. Autoantibodies reacting with native glycophorin A were not detectable in any of the serum fractions examined, as assessed by the lack of Ig bound to column I. In contrast, autoantibodies to asialoglycoprotein (ASG) were present as revealed by binding to column II. This anti-ASG fraction was further purified after elution from column II with 0.1 M N-acetylglucosamine, by vacuum dialysis against buffer I followed by further chromatography using solubilized membrane glycoproteins from untreated subpopulation A cells.

These results parallel the findings obtained in readdition experiments and in agglutination inhibition assays, which also demonstrated the ability of cell-bound Ig to bind to appropriately treated RBC subpopulations and to inhibit PNA-induced agglutination of these cells. In contrast, Ig fractions from autologous sera contained substantial amounts of antibody directed toward ASAGG as well as toward ASG, as indicated by the ability of normal human serum fractions to bind to RBC sequentially treated with VCN and ECG. Nonspecific reaction toward terminal galactosyl residues was ruled out by absorption of anti-ASG antibodies against asialofetuin.

Characterization of the RBC Membrane Ig Binding Site

Peak III, obtained by sequential affinity chromatography of membrane glycoprotein fractions from subpopulation A (old) RBC, had an approximate molecular weight of 50,000 as demonstrated by its elution from a standardized Sephadex G-200 column. This fraction was not readily detectable in SDS polyacrylamide gels, due to the poor staining obtained with Coomassie brilliant blue and PAS staining procedures; however, no contamination of this fraction with other polypeptides was evident. Peak III contained no Ig detectable by DID or RID using goat anti-human IgM, IgA, or IgG but was precipitated in immunodiffusion gels by (A) peaks Ia, Ib, and II (containing IgM, IgA, and IgG, respectively), (B) Ig fractions thermally eluted from subpopulation A RBC, and (C) purified anti-ASG antibodies. Neither purified anti-ASAGG autoantibodies isolated from autologous serum nor purified antibodies directed against M or N blood group determinants precipitated peak III in DID assays (Fig. 4). Peak III was also shown to be a

Table 4. Inhibition of PNA-Induced Agglutination of RBC

<table>
<thead>
<tr>
<th>RBC Subpopulation*</th>
<th>Treatment</th>
<th>PNA Dilution†</th>
<th>Ig Fractions</th>
<th>Anti-ASG§</th>
<th>Anti-ASAGG§</th>
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</thead>
<tbody>
<tr>
<td>A (old)</td>
<td>Heat</td>
<td>512</td>
<td>1,024</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>Heat, VCN</td>
<td>1,024</td>
<td>512</td>
<td>32</td>
<td>512</td>
</tr>
<tr>
<td>B (younger)</td>
<td>VCN</td>
<td>1,024</td>
<td>512</td>
<td>128</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>Heat, VCN</td>
<td>1,024</td>
<td>512</td>
<td>256</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td>ECG, VCN</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>1,024</td>
</tr>
<tr>
<td></td>
<td>Heat, ECG, VCN</td>
<td>256</td>
<td>1,024</td>
<td>512</td>
<td>1,024</td>
</tr>
</tbody>
</table>

*As defined in reference 1.
†Optimum lectin dilution for agglutination, as determined in Table 3.
‡Fractions of Ig eluted from autologous subpopulation A cells by thermal treatment (1), as described in text, separated by Sephadex G-200 chromatography using solubilized membrane glycoproteins from untreated subpopulation A cells.
§Chemically modified glycophorin A and homologous specific autoantibodies obtained from normal human serum.
¶Fraction III from Sephadex G-200, containing the senescent cell antigen.
*Reciprocal of dilution of test fraction in last well with definite agglutination, as described in text.
**Human serum albumin.
powerful inhibitor of PNA agglutinability exhibited both by heat-treated subpopulation A cells (with or without VCN treatment) and by VCN- or VCN/ECG-treated subpopulation B cells (with or without thermal treatment) (Table 4). The heat stability of this fraction was demonstrated by its retention of antigenic and agglutination inhibitory activities after incubation at 56°C for 1 hr.

**DISCUSSION**

Earlier investigations have shown significant age-related alterations in the structure and function of membrane components of human RBC, including decreases in membrane deformability, sialic acid content, phospholipid and cholesterol concentrations, and the activities of several enzymes. Stuart and Cummings have suggested that the elimination of senescent RBC from the peripheral circulation is related to such changes and is mediated by the deposition of normal human serum components. Their hypothesis has received support from this and other studies demonstrating the presence of autologous Ig on the surface of senescent but not younger RBC.

We have investigated two of the parameters associated with the aging of human RBC, namely, the decrease in VCN-susceptible sialyl residues and the deposition of autologous Ig on the surface of senescent RBC. The results of our experiments indicate that younger RBC (subpopulation B) express easily detectable terminal N-acetylneuraminic acid residues on the glycoproteins of their surface membranes, whereas the older, more dense cells (subpopulation A) appear to lack such sialic acid residues. The finding of surface-bound Ig under physiologic conditions only in cells from subpopulation A suggests that Ig-binding sites on the RBC membrane are revealed directly as a result of loss of terminal sialyl residues. Support for this concept is drawn from the observation that VCN treatment of the cells in subpopulation A resulted in the expression of similar binding sites of autologous Ig.

Further characterization of the membrane components involved in the binding of immunoglobulin was provided by sequential enzymatic hydrolysis of intact RBC obtained from subpopulations A and B. Both of these RBC subpopulations released small quantities of terminal galactose residues when treated with ECG, and larger amounts were released by ECG treatment following the elution of surface-bound Ig from subpopulation A cells and after the release of terminal sialyl residues from subpopulation B cells, as assessed both by the quantities of galactose in the supernatants and by the PNA-induced agglutination of treated cells. The binding of Ig previously isolated from the cells in subpopulation A to heat-treated subpopulation A or VCN-treated subpopulation B cells required the presence of terminal galactosyl residues on the cell surface. Since F(ab')2 but not Fe fragments of the specific autoantibodies were similarly bound, the Ig appears to be bound through the Fab portion rather than the Fc portion of the molecule.

The above results suggested the presence of an age-related antigen expressing terminal galactosyl residues on the surface of senescent but not younger human RBC. Attempts were made to isolate this putative antigen, using several procedures based on the affinity of membrane-bound Ig for this RBC membrane component. Membrane glycoprotein frac-
tions from native subpopulation A cells were shown to contain autologous Ig, and the Ig-containing fractions were further purified by affinity chromatography over columns containing immobilized anti-human Ig. Fractionation of bound material on Sephadex G-200 in the presence of chaotropic agents yielded four distinct fractions, of which only three were positive for Ig: peak Ia containing IgM, peak Ib containing IgA, and peak II containing IgG. This Ig was demonstrated to contain "autoantibodies," since all three fractions retained the ability after purification to bind to RBC with terminal galactosyl residues (heat-treated subpopulation A or VCN-treated subpopulation B cells). The fourth peak (III) was shown to contain antigen, since it reacted in DID experiments with all three Ig-containing peaks (Ia, Ib, and II). These findings indicate that the material in peak III represented the age-related antigen expressed on cell membranes of aged RBC. This conclusion was strengthened by the observation that this peak strongly inhibited the PNA-induced agglutination of susceptible RBC bearing terminal galactosyl residues.

Additional characterization of this putative senescent cell antigen (SCA) was carried out by comparison with the previously described major sialoglycoprotein of the RBC membrane.9,24 The specificity of the anti-SCA Ig was examined (A) by solubilization of subpopulation A RBC membranes, (B) by heat elution of native subpopulation A RBC, and (C) by sequential affinity chromatography of normal human serum over specifically modified preparations of glycophorin A. The modifications of glycophorin A included VCN hydrolysis to produce asialoglycophorin (ASG) and VCN/ECG hydrolysis to produce asialoagalactoglycophorin (ASAGG). None of the Ig preparations was reactive with native glycophorin, and only the Ig preparation from normal human serum had anti-ASAGG activity. However, all three Ig preparations bound ASG, confirming the specificity of the eluted Ig for antigens expressing terminal galactose residues and indicating that ASG, as purified by sequential affinity chromatography, was cross-reactive with the senescent cell antigen.

The appearance of asialoglycophorin on senescent but not younger human RBC implies the presence within the normal circulatory system of an enzyme with neuraminidase activity directed against the RBC membrane. This activity would be expected to be short-lived or otherwise regulated to preclude catastrophic autoagglutination in situ. Membrane-bound neuraminidase has been demonstrated by Bosmann25 on the surface membrane of mature but not senescent human RBC. Although other possible explanations for the appearance of an age-related antigen must be considered, including deposition of components normally present in the serum and de novo synthesis of unique membrane determinants, there is no evidence to support the possibility that age-related membrane alterations are due to the binding of such determinants to the RBC surface, and it seems unlikely that senescent RBC, lacking nuclei, would be capable of synthesizing unique determinants de novo. Thus, it is probable that differences between senescent and younger RBC arise as a result of the cleavage of sialic acid residues from the membranes of mature RBC.

The overall effect of age-related alterations in the RBC membrane is an increase in the specific density of the cells.27 In addition, at least one of these changes affects the antigenic properties of the exterior surface of the cell, as evidenced by the presence of autologous Ig bound through the Fab portion of the molecule to the surface antigens of senescent but not younger RBC.1,23 The demonstrated specificity of this Ig binding is consistent with the presence of an adaptive immune response by which senescent RBC are removed from circulation.4 Thus, we postulate that antibodies directed against ASG, which are present in normal individuals, react specifically with antigens exposed on aged RBC, and that antibody-coated senescent (desialylated) cells are then opsonized and subjected to phagocytic removal by reticuloendothelial macrophages.26 Such a mechanism could conceivably control the elimination of other aging cells that normally express MN blood group determinants and, therefore, have the capacity to express this antigen. The reported demonstration of similar cryptic antigens on the surfaces of platelets and leukocytes after neuraminidase treatment27-29 is consistent with such a mechanism. Indeed, it is possible that the senescent cell antigen is present on aged cells of many organs and performs a physiologic role in their removal.

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REFERENCES

Isolation and characterization of an age-related antigen present on senescent human red blood cells

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