Characterization of the Autologous Antibodies That Opsonize Erythrocytes With Clustered Integral Membrane Proteins

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In earlier studies we presented evidence that the clustering of the integral membrane protein, band 3, can serve as a signal for immune recognition and clearance of senescent or abnormal erythrocytes from circulation. In this study, we have exploited the capacity of 1 mmol/L Zn⁺² to mildly and reversibly cluster band 3 in situ to characterize the nature of the autologous antibodies specific for the clustered state. We report that the autologous IgG elute almost exclusively from a high molecular weight complex with other proteins when C₂×E₂ detergent extracts of Zn clustered membranes are chromatographed on Sepharose CL-6B. The complex was also seen to contain complement component C3, hemoglobin, and a cross-linked oligomer of band 3. Autologous IgG and complement were virtually absent from all other fractions. When the band 3 clusters were disaggregated by removal of the Zn⁺², the autologous IgG eluted from the erythrocyte surface. Collection of this IgG and use of the antibody in immunoblots of erythrocyte membranes showed that the band 3 monomer, dimer, and oligomers were the major antigenic species. Except for a minor unidentified band at ~78,000 d, no other proteins were significantly stained. Curiously, band 3 showed an uneven staining pattern, with oligomers and the leading edge of the monomers appearing more intensely than expected from their abundances in the Coomassie blue-stained gels. Typing of the same autologous IgG with monoclonal antibodies specific for the different subclasses of IgG showed the presence of only subtypes 2 and 3. Taken together, these data suggest that a specific population of autologous IgG recognizes sites of integral membrane protein clustering (a common lesion in senescent and abnormal red blood cells) and that the antigen within these clusters involves an aggregated state of band 3.

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HUMAN ERYTHROCYTES commonly circulate for ~120 days and are then captured and destroyed by macrophages of the reticuloendothelial system. Because this recognition and removal process occurs in the absence of de novo protein synthesis, researchers have looked for changes in components of the cell membrane to explain how senescent cells are selectively recognized by macrophages. One hypothesis that has received increasing attention has been that senescent cell clearance is triggered by the clustering of integral membrane proteins, predominantly band 3. In this mechanism, processes such as hemoglobin denaturation, protein oxidation, or protein cross-linking are thought to lead to the aggregation of a few copies of band 3 late in the life of the circulating erythrocyte. As shown previously, this clustering promotes the avid opsonization of the cell by autologous antibodies. Once decorated with antibodies, the cell then accumulates complement, which, together with the antibodies, marks the cell for removal by macrophages. Because the timing of erythrocyte removal is determined by the timing of appearance of defects in the cell's biochemistry, this mechanism allows for the functional integrity of the erythrocyte to determine its own life span.

Recently, in studies aimed at modeling the senescence pathway in vitro, we showed that agents that artificially cluster band 3 (eg, Zn⁺², acridine orange, and melittin) also promote autologous IgG binding, complement component C3 deposition, and phagocytosis of the treated erythrocytes. Because the properties of this artificial senescence/clearance pathway corresponded closely to the characteristics of senescent erythrocyte recognition and removal in vivo, the artificial clustering agents afforded us the opportunity to isolate and characterize large amounts of autologous antibodies that can recognize erythrocytes destined for immediate destruction by macrophages. In this report, we describe the isolation and characterization of these antibodies and their immune complexes. We show that such autologous IgG are tightly bound to macromolecular complexes containing complement component C3, band 3, and hemoglobin. We further show that the eluted IgG recognize band 3 in immunoblots of both unmodified and clustered erythrocyte membranes, and that the antibodies predictably immunoblot cross-linked band 3 more avidly than monomeric band 3.

MATERIALS AND METHODS

Materials: Fresh human blood anticoagulated with heparin (6 IU/mL blood) was used the same day. For a few experiments, blood from the Central Indiana Regional Blood Bank was purchased and used before its expiration date. Affigel and bis(sulfosuccinimidyl)suberate (BS3) were obtained from Pierce (Rockford, IL). Carrier-free Na¹²⁵I was from Amersham International (Arlington Heights, IL) and eosin 5-maleimide was obtained from Molecular Probes (Eugene, OR). Protein A agarose beads were from Pharmacia (Uppsala, Sweden) or Sigma (St. Louis, MO). Rabbit antihuman IgG conjugated to avidin, avidin-horseradish peroxidase, bovine serum albumin, rabbit antihuman C3c antibodies (affinity-purified polyclonals), and mouse monoclonal antibodies specific for the various human IgG subtypes were from Sigma. Antibodies to band 3 were raised in rabbits and affinity purified with immobilized band 3. Human IgG was purchased from ICN (Plainview, NY) and octaethylene glycol mono-n-dodecyl ether (C₈E₈) was from Nikko Chemical Co (Tokyo, Japan). All other reagents were purchased from common commercial sources.

Labeling and clustering of erythrocyte membrane proteins. Erythrocytes were centrifuged at 2,000g to remove the plasma (which...
was saved) and buffy coat, and were washed three times in HEPES-glucose (10 mmol/L HEPES, 130 mmol/L NaCl, 10 mmol/L glucose, pH 7.4). When desired, the cells were incubated for 30 minutes at room temperature in the dark in HEPES-glucose containing 10 μmol/L eosin maleimide to label band 3 in situ. All procedures after eosin maleimide labeling were also performed in the dark. After three washings in HEPES-glucose, the labeled or unlabeled cells were either treated at 10% hemacrit with 1 mmol/L ZnCl₂ plus 1 mmol/L BS3 for 15 minutes at room temperature, or left unmodified. The cross-linked cells were then washed twice in HEPES-glucose-supplemented with 10 mmol/L ethanolamine, pH 7.4, twice in HEPES-glucose, and washed once in HEPES-glucose-supplemented with 10 mg/mL bovine serum albumin (BSA) to remove the Zn²⁺ and BS3. Opsonization was performed at 33% hematocrit by incubation for 60 minutes at 37°C in autologous plasma, as described. For gel filtration chromatography, the autologous plasma was supplemented with autologous radioiodinated human IgG (10 mg/mL) to allow localization of the IgG. Alternatively, in some studies, the serum-equilibrated cells were subsequently labeled with radioiodinated rabbit anti-human IgG. To localize C₃, opsonized cells were washed three times in HEPES-glucose and then incubated in HEPES-glucose supplemented with 10 mg/mL BSA and 0.1 mg/mL radioiodinated antihuman C₃c antibody for 1 hour at 37°C. For all other studies, opsonization was performed in unmodified autologous plasma. After opsonization or treatment with antihuman C₃c, cells were washed five times in HEPES-glucose containing 20 mg/mL BSA and then twice in HEPES-glucose alone. Erythrocyte membranes were then prepared by hemolysis in 5 mmol/L sodium phosphate, 1 mmol/L EDTA, pH 8, and washed until white or until no further hemoglobin could be removed.

**Extraction of membranes with nonionic detergents.** One milliliter of freshly prepared ghosts was mixed with 2 mL extraction buffer (10 mmol/L HEPES, 130 mmol/L NaCl, 10 mmol/L N-ethyl maleimide, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1.5% C₁₂E₈, pH 7.4) to yield a final detergent concentration of 1%. When other detergents were used (eg, Triton X-100 or polydocanol), an artificial aggregation of membrane proteins gradually ensued, even in the absence of ZnCl₂. For this reason, only the highly purified C₁₂E₈ was used. After the addition of extraction buffer, the membranes were agitated by moderate shaking for 20 minutes at 37°C and then pelleted for 1 minute at 13,000 rpm in an Eppendorf microfuge. The clear supernatant was immediately separated from the pellet and used for gel filtration chromatography or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Gel filtration of detergent-extracted membrane proteins on Sepharose CL-6B.** A 100 × 1.7 cm column was filled with Sepharose CL-6B and equilibrated with a buffered solution containing 50 mmol/L NaCl, 10 mmol/L HEPES, 0.1% C₁₂E₈, pH 7.4. Three milliliters of C₁₂E₈ detergent-extracted membrane proteins was then loaded onto the column and separated at a flow rate of 0.9 mL/min. Constant flow was maintained by using a Waters high performance liquid chromatography (HPLC) pump (Waters, Milford, MA). The effluent was collected in 4.5 mL fractions. For electrophoretic analysis, samples were concentrated ten times by vacuum centrifugation and then diluted 1:1 with 4X concentrated sample buffer and incubated 30 minutes at room temperature.

**Isolation of clustering-dependent autologous IgG.** Erythrocytes were suspended at 10% hematocrit for 15 minutes at 37°C in HEPES-saline containing 1 mmol/L ZnCl₂ to cluster band 3. After pelleting, 10 mL of the packed red blood cells was mixed with 10 mL HEPES saline containing 3 mmol/L ZnCl₂ plus 10 mL autologous serum to permit opsonization of the Zn²⁺-clustered cells. After 1 hour of incubation at 37°C, the cells were washed three times in HEPES-saline supplemented with 1% BSA and 1 mmol/L ZnCl₂, and again for three times in HEPES-saline supplemented with 1 mmol/L ZnCl₂. By this method, the Zn²⁺-clustered state was maintained throughout all opsonization and washing steps. To the washed red blood cell pellet was then added 10 mL HEPES-saline lacking ZnCl₂, and the suspension was incubated and mildly agitated for 5 minutes at room temperature to allow dissociation of clustering-dependent autologous IgG. The cells were pelleted at 3,000g and the supernatant was saved. This elution protocol was repeated five times and the collected supernatants were pooled. The autologous IgG fraction was then either used directly for Western blotting or concentrated by precipitation for 1 hour at 4°C in 10% trichloroacetic acid following by washing with cold acetone and used later for IgG subtype analysis.

**Characterization of isolated autologous IgG.** The acetone-washed IgG precipitate described above was separated on 5% to 15% polyacrylamide gels according to the method of Laemmli and transferred to nitrocellulose, as described. After blocking for 3 hours in 3% gelatin, mouse monoclonal antibodies to the various IgG subtypes were added and then horseradish peroxidase conjugated to goat antimouse IgG was administered. The blots were then developed with 4-chloronaphthol.

Identification of the red cell blood antigens recognized by the clustering-dependent IgG was accomplished by separating Zn²⁺/BS3-treated and unmodified erythrocyte membranes on SDS 5% to 15% polyacrylamide gradient gels and blotting the separated proteins onto nitrocellulose. Visualization of the recognized antigens was performed by staining with the eluted autologous IgG described above. Briefly, after blocking with 3% gelatin, the blots were incubated overnight at 4°C in the eluted IgG solution supplemented with gelatin. The blots were then developed by 3 hours of incubation in biotinylated antihuman IgG followed by 2 hours of incubation in avidin-horseradish peroxidase and then 4-chloronaphthol.

**Immunoprecipitation with protein A.** Fifty microliters of protein A agarose beads (Sigma, Pharmacia) was washed in HEPES buffer supplemented with 0.1% C₁₂E₈, pH 7.4, added to 500 μL detergent-extracted membrane proteins, and incubated at 4°C for 3 hours under shaking. The beads were then washed six times in HEPES buffer supplemented with 0.1% C₁₂E₈, pH 7.4, and added to 50 μL 5X sample buffer. After 30 minutes of incubation at room temperature, the bead supernatant was removed, separated by SDS-PAGE, and immunoblotted, as detailed above, by using horseradish peroxidase-conjugated antihuman IgG, rabbit antihuman C₃c, and horseradish peroxidase-conjugated antirabbit IgG antibodies.

**Immunoprecipitation with anti-C₃c antibodies.** Ten milligrams of antihuman C₃c antibodies was bound to 500 μL of Affi-gel beads according to the manufacturer’s (Bio Rad, Richmond, CA) instructions. Two hundred microliters of beads was added to 1 mL of pooled fractions 15 to 17 (Fig 1, peak A) obtained by gel filtration of the detergent-extracted membrane proteins from Zn²⁺/BS3-treated cells. After shaking, the beads were washed six times in HEPES buffer, pH 7.4, and added to 50 μL 5X electrophoresis sample buffer. After 30 minutes of incubation at room temperature, the bead supernatant was used for SDS-PAGE and immunoblotting as detailed above.

**RESULTS**

Isolation of membrane protein complexes containing autologous IgG. In previous work, we and others have shown that Zn²⁺ can nonoxidatively and reversibly cluster band 3 in the plane of the membrane of intact erythrocytes. When desired, this clustered distribution can also be stabilized with the impermeable cross-linker, BS3, allowing the removal of the Zn²⁺ without loss of the induced affinity for autologous IgG. In the absence of Zn²⁺, BS3 was unable to promote
autologous IgG or complement binding. To begin to identify the membrane components recognized by the autologous IgG, fresh human erythrocytes were treated with Zn$^{2+}$ and BS3, incubated in their own serum, washed extensively, hemolyzed in buffer to prepare ghosts, and then extracted with a clean nondenaturing detergent (1.0% \( \text{C}_{12} \text{E}_{8} \)) to solubilize membrane proteins (see Materials and Methods). After pelleting out the membrane skeletons, the supernatant was chromatographed on a Sepharose CL-6B gel filtration column and fractions were analyzed for IgG, complement, and other erythrocyte components. As shown in Fig 1a, the Zn$^{2+}$/BS3 treatment shifted material absorbing at 280 nm from fractions 23 to 39 (peaks B and C) to higher molecular weight, predominantly into peak A. This redistribution of protein into the excluded volume fraction is consistent with the ability of Zn$^{2+}$ to mildly cluster band 3 and the ability of BS3 to fix this clustered distribution.$^{3,15}$

The localization of eosin 5-maleimide fluorescence (a specific label for band 3$^{15}$) (Fig 1b) indicated that in unclustered cells band 3 is predominantly localized in peak B, with a minor fraction of very high molecular weight in peak A. Because unmodified band 3 is thought to consist of a mixture of band 3 dimers and tetramers, we presume that both oligomeric forms migrate in peak B. In contrast to unmodified cells, a significant fraction of band 3 from Zn$^{2+}$/BS3-clustered erythrocytes is measurably shifted from peak B to peak A.
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Fig 2. Immunoblots of immune complexes collected from detergent extracts of Zn\textsuperscript{2+}/BS3-treated membranes. Detergent extracts of membranes from opsonized Zn\textsuperscript{2+}/BS3-treated cells were analyzed directly by electrophoresis and immunoblotting (lanes A, C, and E), or incubated with protein A-Sepharose beads to collect immune complexes and analyzed by electrophoresis and immunoblotting (lanes B, D, and F). Nitrocellulose blots were developed with antibodies to band 3 (lanes A and B), to human IgG (lanes C and D), or to complement component C3c (lanes E and F) (see Materials and Methods for details). Band 3 monomer and dimer migrate at apparent molecular weights of ~95,000 and 190,000, IgG at ~160,000, and complement C3 at ~200,000. The antibody to band 3 is directed against the cytoplasmic domain and recognizes numerous proteolysis products that arise in the extract during brief storage.

In summary, the column profiles in Fig 1 would suggest that a fraction of the cellular band 3, along with IgG, complement, and hemoglobin, is present in the same macromolecular aggregate.

Confirmation that autologous IgG, complement, band 3, and hemoglobin are present in the same membrane complex. To confirm that complement, band 3, and hemoglobin are present in the same complex with autologous IgG, it was decided to collect the immune complexes on immobilized protein A and evaluate whether complement, band 3, and hemoglobin might also coisolate. Figure 2 displays a comparison of the nitrocellulose blots of an electrophoretically separated C\textsubscript{13}E\textsubscript{4} membrane extract of Zn\textsuperscript{2+}/BS3-treated cells with analogous blots of the immune complexes isolated from the same detergent extract. As expected, those lanes stained with antibodies to band 3 showed a greater abundance of band 3 in the total detergent extract (lane A) than in the immunoprecipitate (lane B). This is because the vast majority of band 3 is not involved in the IgG-containing aggregate, as seen in the elution profile in Fig 1b. In contrast, the relative concentration of autologous IgG is much greater in the immunoprecipitate (lane D) than it is in the unfractionated extract (lane C), confirming that the protein A beads indeed avidly collect the immune complexes from the membrane extract. However, immunoblots of analogous immune complexes from either opsonized unclustered cells or nonopsonized Zn\textsuperscript{2+}/BS3-treated cells did not show reactivity with antibodies to either band 3 or human IgG (not shown). Thus, to form immune complexes, both band 3 clustering and serum incubation must be required. Evidence that comple-
ment component C3 is also present in the complexes was obtained by staining the same blots with antibodies to C3. As seen in lanes E and F, the complement component was enriched in the immune complexes over the unfractionated membrane extract. The simplest explanation of both immunoprecipitation and Sepharose CL-6B chromatography studies is that band 3, IgG, and complement are present in the same membrane aggregates. Furthermore, because the protein A-containing beads appeared distinctly red after incubation in the detergent extracts, we conclude that hemoglobin is also present in the complex.

It should be noted that the anti-band 3 staining component in the immune complexes in lane B of Fig 2 extends to higher molecular weight than the average band 3 in either the detergent extract of control membranes (not shown) or of Zn\(^{2+}\)/BS3-treated membranes (lane A). This abnormal molecular weight distribution implies that autologous antibodies opsonize membrane sites containing an enrichment of aggregated band 3. Immunoblots of the high molecular weight peak (fraction A) from the gel filtration column also displayed a distinct enrichment of cross-linked band 3 (data not shown). However, whether cross-linked band 3 constitutes the antigen recognized by the autologous IgG cannot be deduced from these data.

**Specificity of the autologous IgG eluted from Zn\(^{2+}\)-clustered cells.** To determine the identity of antigens recognized by autologous antibodies on Zn\(^{2+}\)-clustered cells, clustering-dependent antibodies were eluted from Zn\(^{2+}\)-treated erythrocytes simply by removal of the Zn\(^{2+}\) (see Materials and Methods). In the absence of fixation with BS3, removal of Zn\(^{2+}\) allows the clustered proteins to dissociate and, thereby, destroys the high-affinity binding site for autologous IgG. IgG that were collected in this manner were subsequently used to immunoblot both Zn\(^{2+}\)/BS3-treated and unmodified membranes to try to identify the components recognized by the autologous IgG. As shown in the Coomassie blue–stained lanes of Fig 3, Zn\(^{2+}\)/BS3 treatment shifts a fraction of the band 3 from its normal position in the gel to positions anticipated for dimeric and tetrameric band 3 (compare lanes A and B). Consistent with the data of Fig 1B, this fraction is very small. Except for the appearance of aggregated material at the top of the gel, no other major changes are stabilized by the impermeant BS3. When the Zn\(^{2+}\)/BS3-treated and control membranes are then immunoblotted with autologous IgG eluted from Zn\(^{2+}\)-clustered cells, all major band 3 species are visualized (Fig 3, lanes C and D). Thus, in the clustered membranes (lane C), species corresponding to the band 3 monomer, dimer, tetramer, and polymer are all stained, whereas in the control membranes only the monomer and some dimer are detected (lane D). In some control samples, only monomeric band 3 could be seen in the immunoblot (data not shown). In contrast, antibodies eluted from unclustered membranes were unable to stain any polypeptides in either Zn\(^{2+}\)/BS3-treated (lane E) or unmodified (lane F) membranes.

Although the cluster-dependent autologous IgG recognized all major oligomeric states of band 3, their staining intensities did not correspond to the relative abundances of each form of band 3 in the gel. Thus, the tetramer and dimer stained considerably more intensely with the eluted autologous antibody than with a rabbit polyclonal antibody to human band 3 (data not shown), and the leading edge of the monomer in band 3 stained more vigorously with autologous IgG than expected from the Coomassie blue staining pattern (compare lanes A and C). We hypothesize that the former disproportionate staining might be attributable to a preference of the eluted antibodies for clustered band 3, whereas the latter selectivity may suggest that an underglycosylated form of band 3 is more easily recognized than its more heavily glycosylated counterpart. However, other explanations of this staining pattern are also possible.

**IgG subtype of clustering-dependent autologous IgG.** There are four major classes of IgG that differ in the sequences of their heavy chains. Because autologous antibodies represent a special fraction of IgG responsible for elimination of autologous antigens, it was conceivable that they might derive from a single subtype of IgG. To examine this possibility, clustering-dependent IgG were separated electrophoretically under nonreducing conditions, blotted
onto nitrocellulose, and stained with antibodies to the four major subtypes of IgG. As shown in Fig 4, only types 2 and 3 appear to be represented in the clustering-dependent IgG. Although type-2-specific IgG stained the eluted antibody (lane 3) somewhat more intensely than did type-3-specific IgG, it also stained the unfraccionated serum more avidly (lane 4), consistent with the fivefold higher concentration of type 2 IgG in the serum. However, because type 1 IgG is clearly the most abundant subtype in whole serum, its absence from the autologous IgG pool suggests that some specificity in the biosynthesis of this functional class of IgG exists.

DISCUSSION

In previous work we have presented evidence that autologous IgG that recognize integral membrane protein aggregates are involved in the clearance of abnormal and senescent erythrocytes from circulation.\textsuperscript{6,10,11} In the present study, we have attempted to characterize these antibodies by inducing the clustered state with 1 mmol/L ZnCl\textsubscript{2}. Our data show that such autoantibodies bind almost exclusively to membrane protein aggregates containing band 3, hemoglobin, and complement component C3. The presence of other membrane proteins in these clusters was not examined in this study; however, previous publications confirm that lesser quantities of spectrin, ankyrin, band 4.1, and actin are also coaggregated.\textsuperscript{6,8,10,11} The immunoblots in Fig 3, nevertheless, show that band 3 is the predominant antigen recognized by the IgG, and that not all forms of band 3 are comparably opsonized. The preference of autologous IgG for covalently cross-linked/aggregated band 3 may be consistent with the strong dependence of IgG binding on band 3 clustering.\textsuperscript{2,3}

Why would an antibody preferentially recognize a clustered antigen over a dispersed form of the same polypeptide? The answer may be that a bivalent attachment of antibody to two adjacent antigens occurs with twice the free energy change (\(\Delta G\)) of a single attachment. Because the equilibrium binding constant of the antibody is related exponentially to this \(\Delta G\), the resulting bivalent binding constant would be roughly the product of the two monovalent constants. Actually, when the lower entropy decrease of the second arm attachment is considered, the net binding constant can be even greater.\textsuperscript{2} Thus, a weakly binding antibody that cannot form a stable complex with a monovalent site might bind very avidly when the monovalent sites are aggregated. We have proposed such a mechanism for the selective recognition of band 3 in senescent and abnormal erythrocytes\textsuperscript{2} because a minor fraction of the band 3 in these cells is frequently clustered.\textsuperscript{6,8,10,11}

Although autologous antibody-enriched aggregates from senescent/abnormal erythrocytes have never been analyzed by gel filtration chromatography, they still appear to be very similar to the aggregates characterized in this model study. Thus, they are enriched in band 3 and globin and, at least in the case of \(\beta\)-thalassemic cells, they contain elevated levels of complement component C3.\textsuperscript{6,10,11} Furthermore, although the aggregates in sickle cells,\textsuperscript{10} \(\beta\)-thalassemic cells,\textsuperscript{11} and the densest fraction (0.5%) of normal cells\textsuperscript{6} constitute only 1.3%, 1.6%, and 0.09% of the total membrane protein, respectively, they still contain 75%, 27%, and 55%, respectively, of the total cell surface autologous IgG. Thus, as with Zn\textsuperscript{2+}-treated cells, the membrane protein aggregates in naturally circulating cells represent the predominant site of autologous IgG binding. It would be interesting to learn whether antibodies eluted from sickle cells, \(\beta\)-thalassemic cells, or the dense fraction of normal cells exhibit elevated affinity for cross-linked band 3. In the case of normal cells, at least one report suggests that they do.\textsuperscript{16}

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