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Binding of Anti-Band 3 Autoantibody to Sialylated Poly-*N*-Acetyllactosaminyl Sugar Chains of Band 3 Glycoprotein on Polyvinylidene Difluoride Membrane and Sepharose Gel: Further Evidence for Anti-Band 3 Autoantibody Binding to the Sugar Chains of Oxidized and Senescent Erythrocytes

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Binding specificity of naturally occurring anti-band 3 IgG antibody isolated from human plasma was investigated in a cell-free binding system. ^{125}I-labeled human anti-band 3 IgG specifically bound to band 3 glycoprotein and lactoferrin, a glycoprotein that has poly-*N*-acetyllactosamine-type sugar chains like band 3, on the polyvinylidene difluoride blotting membrane. Binding was decreased by 50-70% when band 3 and lactoferrin were pretreated with *N*-glycosidase F, endo-β-galactosidase, or neuraminidase. Binding of ^{125}I-anti-band 3 IgG to band 3-Sepharose gel was partially inhibited by band 3 oligosaccharides or lactoferrin, but was less inhibited by them after they had been treated with *N*-glycosidase F or endo-β-galactosidase. A significant part of ^{125}I-anti-band 3 IgG that bound to the band 3-Sepharose gel was released upon treatment of the gel with *N*-glycosidase F or endo-β-galactosidase. IgG that binds to lactoferrin (anti-lactoferrin IgG) was isolated from normal human plasma. ^{125}I-Anti-lactoferrin IgG bound to the band 3-Sepharose gel as effectively as to the lactoferrin-Sepharose. The antibody specifically bound to the band 3- and lactoferrin-blotted membrane depending on the poly-*N*-acetyllactosaminyl sugar chains of the blotted glycoproteins. The results indicate that a major part (about 70%) of anti-band 3 IgG recognizes the sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3 and lactoferrin, and the remaining part (about 30%) of the antibody may recognize the polypeptide portion of band 3. This was supported by the observation that anti-band 3 IgG effectively bound to lactoferrin-Sepharose but 33% of the antibody did not. Anti-band 3 IgG with the carbohydrate-binding property was equally obtained whether fully denatured or barely denatured band 3 was used for isolation of anti-band 3 IgG by affinity chromatography. These results provide further evidence for our proposal that the binding sites of anti-band 3 IgG to oxidized and senescent erythrocytes reside on the locally condensed sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3 on the cell surface.

Key words: anti-band 3 autoantibody, band 3 glycoprotein, lactoferrin, senescent cell antigen, sialylated poly-*N*-acetyllactosaminyl sugar chain.

Senescent erythrocytes are removed from the blood circulation by the reticuloendothelial system (*1, 2*). There are natural IgG antibodies that bind selectively to senescent erythrocytes in human plasma, and binding of the antibodies to the cells has been implicated in causing Fc receptor-mediated phagocytosis of the senescent cells by macrophages of the reticuloendothelial system (*3-5*). Several studies have demonstrated the involvement of binding of anti-band 3 IgG autoantibody to band 3 glycoprotein on the surface of senescent erythrocytes (*6-9*). Thus, anti-band 3 IgG appears to play an important role in the removal of senescent erythrocytes from the circulation.

Two different mechanisms have been presented for the appearance of the antigen on senescent erythrocytes (senescent cell antigen) to which anti-band 3 IgG binds: formation of band 3 fragments by polypeptide cleavage (*6-8*) and clustering of band 3 molecules (*10-14*). Furthermore, localization of the antigenic determinants on band 3 molecule has been attempted by three groups. We have shown, in a series of investigations on binding of anti-band 3 IgG to oxidized (*15-17*) and senescent erythrocytes (*18*), that the antigenic determinants of band 3 are located in its sialylated poly-*N*-acetyllactosaminyl sugar chains. On the contrary, Kay *et al.* (*19*) and Lutz *et al.* (*20, 21*) have reported that the antigenic determinants are present on the polypeptide portion of the glycoprotein.

The present study was undertaken to clarify, in a cell-

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Abbreviations: BSA, bovine serum albumin; $C_{12}E_8$, octaethylene glycol *n*-dodecyl monoether; DPBS, Dulbecco's phosphate-buffered saline; DPBS(−), Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

free system, whether anti-band 3 IgG binds to the sugar chains of band 3. Band 3 and human lactoferrin, a sialylated poly-*N*-acetyllactosaminyl sugar chain-containing glycoprotein, were adsorbed on polyvinylidene difluoride (PVDF) blotting membrane or covalently bound to Sepharose gel, and binding of ^{125}I-labeled human anti-band 3 IgG to the membrane or the gel was investigated. It was found that major binding sites in band 3 for anti-band 3 IgG reside in its sialylated poly-*N*-acetyllactosaminyl sugar chains. The present results give further evidence for our previous proposal (*17, 18*) that binding sites of anti-band 3 IgG on oxidized or senescent erythrocytes reside in the sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3 glycoprotein on the cell surface.

MATERIALS AND METHODS

Materials—Globulin-free bovine serum albumin (BSA) and lactoferrin (from human milk) were obtained from Sigma Chemical (St. Louis, MO). Highly specific *N*-glycosidase F [EC 3.5.1.52, recombinant peptide-N^4-(acetyl-β-glucosaminyl) asparagine amidase cloned from *Flavobacterium meningosepticum* and expressed in *Escherichia coli*] was obtained from Boehringer Mannheim. Endo-β-galactosidase [EC 3.2.1.103] from *Escherichia freundii* was obtained from Seikagaku Kogyo (Tokyo). The preparation specifically hydrolyzes the β-galactosidic linkages of galactosyl residues in glycoproteins bearing the structure-GlcNAcβ1-3Galβ1-4Glc (or GlcNAc). Both *N*-glycosidase F and endo-β-galactosidase were found to contain no protease activity when ^{125}I-labeled human IgG was treated with these enzyme preparations under the conditions employed in the present experiments and the mixture was analyzed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and radioluminography (*18*). Neuraminidase [EC 3.2.1.18] from *Vibrio cholerae* was from Behringwerke AG (Marburg, Germany).

Na^{125}I was obtained from ICN Pharmaceutical (Irvine, CA). Clear Blot Membrane-P, a polyvinylidene difluoride (PVDF) microporous membrane for protein blotting, was obtained from Atto (Tokyo). Sepharose 4B was from Pharmacia LKB Biotechnology (Uppsala, Sweden). Octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$) was from Nikko Chemical (Tokyo). Other reagents were obtained from Wako Pure Chemical (Osaka).

Band 3 and Its Oligosaccharides—Band 3 was isolated from blood group O normal adult human erythrocyte membrane according to the method of Tsuji *et al.* using SDS (*22*) followed by removal of dodecyl sulfate by complexing with K^+ as described previously (*15*) or by the method of Casey and Reithmeier using $C_{12}E_8$ (*23*). The preparation isolated by the former method was routinely used unless otherwise stated. Band 3 oligosaccharides were obtained by hydrazinolysis of the isolated membrane protein fractions according to the methods previously described (*18*). The oligosaccharide preparation contained only a negligible amount of peptides as determined by amino acid analysis. The amount of the oligosaccharides was expressed as a Glc equivalent determined by the phenol/sulfuric acid method (*24*).

Enzymatic Treatment of Band 3, Band 3 Oligosaccharides, and Lactoferrin—Band 3 (1 mg/ml) or lactoferrin (1 mg/ml) was treated with *N*-glycosidase F (1 unit/ml) in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline [DPBS(−)] containing 0.02% NaN_3 at 37°C for 96 h, in order to cleave the *N*-glycosidic linkage of the glycoprotein.

Band 3 (1 mg/ml), band 3 oligosaccharides (2 mg/ml), or lactoferrin (1 mg/ml) was treated with endo-β-galactosidase (100 milliunits/ml) in 0.1 M sodium acetate buffer, pH 5.8, containing 0.07 M NaCl and 0.02% NaN_3 at 37°C for 96 h, in order to cleave the poly-*N*-acetyllactosamine structure of the sugar chain.

Band 3 (1 mg/ml) or lactoferrin (1 mg/ml) was treated with neuraminidase (100 milliunits/ml) in Dulbecco's phosphate-buffered saline (DPBS) containing 0.02% NaN_3 at 37°C for 96 h, in order to cleave the glycosidic bond of the terminal sialic acid.

When the band 3 preparation purified by the method of Casey and Reithmeier (*23*) was digested, 0.1% $C_{12}E_8$ was present throughout the procedure.

Band 3- and Lactoferrin-Sepharose Gels—Immobilized band 3- and lactoferrin-Sepharose gels were prepared as described (*15*). To a 6 ml of Sepharose 4B gel activated with CNBr, 8 ml of a solution of band 3 (5.6 mg/ml), either in SDS-depleted solution (for the band 3 isolated using SDS) or in 0.1% $C_{12}E_8$, or lactoferrin (2.5 mg/ml) in 0.1 M $NaHCO_3$, pH 9.0, was added. To prepare the control gel, the gel was similarly treated without the glycoprotein. The mixture was stirred at 4°C for 12 h. The gel was recovered by centrifugation at $1,500 \times g$ for 10 min, and was successively washed with 12 ml of a solution of 0.3 M monoethanolamine-0.15 M $NaHCO_3$-0.15% Triton X-100 and with DPBS(−) containing 0.02% NaN_3. The gel was resuspended in DPBS(−) containing 0.02% NaN_3 for use. Approximately 5.3 mg of band 3 and 2.1 mg of lactoferrin were found to be coupled per 1 ml of wet gel, when the amount of uncoupled glycoprotein was determined by the Lowry method (*25*).

Anti-Band 3 IgG and Anti-Lactoferrin IgG—Anti-band 3 IgG was obtained from the IgG fraction of blood group AB normal adult plasma by affinity chromatography through a column of band 3-Sepharose 4B gel according to the method of Lutz *et al.* (*20*) after having been passed through a column of Sepharose 4B gel to remove any IgG population adherent to the gel. Anti-lactoferrin IgG was similarly obtained from the same plasma by affinity chromatography through a column of lactoferrin-Sepharose gel. The concentration of IgG was determined using E (1%, 1 cm) 280 nm = 14.0 (*26*).

^{125}I-Radiolabeling of anti-band 3 IgG and anti-lactoferrin IgG was carried out according to the chloramine T method (*27*). Thus, anti-band 3 IgG (25.5 μg) or anti-lactoferrin IgG (25 μg) was reacted with 40 μCi of Na^{125}I, and a solution of ^{125}I-anti-band 3 IgG (8 μg/ml) or ^{125}I-anti-lactoferrin IgG (5 μg/ml) in DPBS(−) containing 0.02% NaN_3 was obtained. Specific activities of ^{125}I-anti-band 3 IgG and ^{125}I-anti-lactoferrin IgG were 7×10^4 and 2.3×10^3 cpm/μg, respectively.

Binding of ^{125}I-Anti-Band 3 IgG or ^{125}I-Anti-Lactoferrin IgG to a PVDF Blotting Membrane Adsorbed with Band 3 or Lactoferrin—A 400-μl solution of BSA (control), band 3, glycosidase-treated band 3, lactoferrin or glycosidase-treated lactoferrin (250 μg/ml, each) in DPBS(−) containing 0.02% NaN_3 was loaded onto a PVDF blotting membrane set in a blotting apparatus (Sanplatec, Osaka) through a slot (10-mm diameter) over the membrane, and

the loaded membrane was allowed to stand at room temperature for 10 min. Then the glycoprotein was adsorbed on the membrane by aspiration. Each membrane was incubated in 5 ml of DPBS(−) containing 1% BSA and 0.02% NaN_3 at 4°C overnight, and washed three times with 20 ml of DPBS(−) for 5 min.

The amount of glycoprotein bound to the blotting membrane under the conditions employed was estimated as follows. Band 3 and lactoferrin were labeled with ^{125}I as described, and they were treated with glycosidases as described. The labeled glycoprotein (100 μg) was adsorbed on the blotting membrane in the same fashion as described above and the radioactivity of the membrane was measured by radioluminography.

The membrane was gently swirled in 5 ml of DPBS(−) containing 0.3 μg/ml of ^{125}I-anti-band 3 IgG or 2.5 μg/ml of ^{125}I-anti-lactoferrin IgG, 1% BSA, and 0.02% NaN_3 at 4°C for 4 h (for ^{125}I-anti-band 3 IgG) or for 20 h (for ^{125}I-anti-lactoferrin IgG). The membrane was washed 5 times by shaking with 10 ml of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.01% Triton X-100 for 15 min. The membrane was air-dried, and the radioactivity bound to the membrane was measured by radioluminography using a Fujix BAS 2000 Bio-Imaging Analyzer (Fuji, Tokyo).

Binding of ^{125}I-Anti-Band 3 IgG to Band 3 Western Blots—Band 3 or glycosidase-treated band 3 was electrophoresed in the presence of SDS by the method of Laemmli (*28*) using 10% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250 (CBB) or subjected to Western blotting according to the method of Towbin *et al.* (*29*) with minor modifications using PVDF membrane. Binding of ^{125}I-anti-band 3 IgG to the Western blots was carried out as described above for binding of ^{125}I-anti-band 3 IgG to the band 3-blotted PVDF membrane.

Binding of ^{125}I-Anti-Band 3 IgG and ^{125}I-Anti-Lactoferrin IgG to Band 3- and Lactoferrin-Sepharose Gel—^{125}I-Anti-band 3 IgG or ^{125}I-anti-lactoferrin IgG, which were purified by affinity chromatography through a column of the band 3-Sepharose or the lactoferrin-Sepharose gel, respectively, was applied to a column of the band 3-Sepharose, the lactoferrin-Sepharose or the control Sepharose gel (0.5 ml) equilibrated with DPBS(−) containing 1% BSA and 0.02% NaN_3. The column was kept at 4°C for 4 h. The column was then eluted with 1.0 ml of DPBS(−), and the eluate was repeatedly passed through the column. The column was eluted successively with 2.0 ml of DPBS(−) and 10.0 ml of 0.1 M glycine-HCl buffer, pH 2.6. Each 0.5-ml fraction was collected and the radioactivity of the fraction was measured using an Aloka ARC-2000 autowell gamma counter.

Inhibition of Binding of ^{125}I-Anti-Band 3 IgG to Band 3-Sepharose Gel by Band 3 Oligosaccharides and Lactoferrin—The band 3-Sepharose gel and the control gel were precoated with BSA by rinsing in DPBS(−) containing 1% BSA at 4°C overnight. A 25-μl aliquot of the wet gel was incubated in 200 μl of supernatant of a preincubated mixture of equal volumes of ^{125}I-anti-band 3 IgG (0.3 μg/ml) and the solution of band 3 oligosaccharides (1.5 mg/ml) or lactoferrin (1.0 mg/ml), at 4°C for 20 h in a micro polypropylene centrifugation tube with a filter (10×40 mm) (Ultrafree C3-GV, Millipore, Tokyo) coated with BSA. The gel was recovered on the filter by centrifugation at 2,000×*g* for 5 min. The gel was washed 4 times by centrifugation with 400 μl of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.01% Triton X-100. Radioactivity of the whole gel cake was counted. After the radioactivity measurement, the gel cake was suspended in 2 ml of 2 N HCl, then the suspension was heated at 100°C for 1 h to hydrolyze the gel, and the amount of sugars in the hydrolysate was determined by the phenol/sulfuric acid method (*24*). Radioactivity was corrected on the basis of 420 μg Gal equivalents (25 μl of wet Sepharose gel).

Release of ^{125}I-Anti-Band 3 IgG from Band 3-Sepharose Gel—Band 3-Sepharose gel (25 μl) incubated with 100 μl of a solution of ^{125}I-anti-band 3 IgG (0.3 μg/ml) in a BSA-coated tube as described was washed 4 times with 400 μl of DPBS(−). Radioactivity of the gel was counted to estimate the amount of the bound ^{125}I-anti-band 3 IgG. The gel in the tube was incubated in 100 μl of a solution of *N*-glycosidase F (1 unit/ml) in DPBS(−) containing 0.02% NaN_3 or 100 μl of a solution of endo-β-galactosidase (100 milliunits/ml) in 0.1 M sodium acetate buffer, pH 5.8, containing 0.07 M NaCl and 0.02% NaN_3 at 37°C for 4 h. The gel was recovered by centrifugation at 2,000×*g* for 10 min, and was washed once with 400 μl of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.01% Triton X-100. Radioactivity of the gel and the combined filtrates was counted to estimate the amount of ^{125}I-anti-band 3 IgG remaining in and released from the gel, respectively.

RESULTS

Binding Specificity of Anti-Band 3 IgG Isolated by Affinity Chromatography Using a Band 3 Column—Band 3 was isolated from human erythrocytes after solubilization of the glycoprotein using SDS as described (*22*), and naturally occurring anti-band 3 IgG was isolated from normal human IgG fraction according to the method of Lutz *et al.* (*20*) by affinity chromatography through a column of Sepharose gel covalently coupled with band 3. The yield of the antibody was 0.03–0.06% of the total IgG.

Band 3 was adsorbed on a protein blotting membrane composed of PVDF. As a control, BSA was similarly adsorbed on the membrane. Assay of anti-band 3 IgG binding to the band 3-blotted membrane was performed by incubation of the membrane with the ^{125}I-labeled anti-band 3 IgG at 4°C for 4 h. After washing of the membrane, radioactivity bound to the membrane was measured by radioluminography. Radioactivity bound to the membrane containing band 3 was much higher than that bound to the control BSA-blotted membrane (nonspecific binding), indicating that binding of anti-band 3 IgG to the band 3-blotted membrane is mostly specific (Fig. 1A). The specific binding of ^{125}I-anti-band 3 IgG to the band 3-blotted membrane was assessed by subtracting the radioactivity bound to the control BSA-blotted membrane. When band 3 was pretreated with *N*-glycosidase F, an enzyme that specifically cleaves the *N*-glycosidic bonds between the oligosaccharide and the polypeptide chains of band 3, binding of radioactivity was diminished (31.2±9.6% of the specific binding to untreated band 3) (Fig. 1A). Hence, the sugar chains of the glycoprotein were suggested to participate in binding of anti-band 3 IgG to band 3. When band 3 was pretreated with endo-β-galactosidase, an enzyme that specifically cleaves poly-*N*-acetyllactosaminyl sugar chains in band 3 (*30*), binding of radioactivity was decreased

similarly to that to the *N*-glycosidase F-treated band 3 (35.8±7.0% of the specific binding to untreated band 3) (Fig. 1A). The result indicates that the poly-*N*-acetyllactosaminyl sugar chains of band 3 are involved in binding of anti-band 3 IgG. When band 3 was pretreated with neuraminidase, binding of radioactivity was also decreased (44.2±13.4% of the specific binding to untreated band 3) (Fig. 1A), indicating the importance of sialyl residues of the poly-*N*-acetyllactosaminyl chains of band 3 in binding of anti-band 3 IgG.

It is known that human lactoferrin, a glycoprotein present in milk and plasma, has several types of *N*-linked oligosaccharide chains including sialylated poly-*N*-acetyllactosamine-type chains (sialylated di-*N*-acetyllactosaminyl chains) (*31*). Lactoferrin was adsorbed on the blotting membrane, and binding of ^{125}I-anti-band 3 IgG to the lactoferrin-blotted membrane was examined (Fig. 1B). ^{125}I-Anti-band 3 IgG effectively bound to the lactoferrin-blotted membrane as compared with the binding to the control BSA-blotted membrane, and pretreatment of lactoferrin with *N*-glycosidase F, endo-β-galactosidase, or neuraminidase decreased the binding (41.3±3.2, 39.4±3.5, and 48.6±1.9% of the specific binding to untreated lactoferrin, respectively). The result shows the involvement of the sialylated di-*N*-acetyllactosamine structure of lactoferrin in the IgG binding. From these results it is very likely that at least 60–70% of anti-band 3 IgG is antibody directed to the carbohydrate chains of band 3.

To confirm the presence of antibody to the poly-*N*-acetyllactosaminyl sugar chains of band 3 in the anti-band 3-IgG preparation, the binding specificity was investigated further by binding assay using band 3-Sepharose gel. ^{125}I-Anti-band 3 IgG was incubated with band 3-Sepharose gel in the absence or presence of band 3 oligosaccharides isolated from erythrocyte membrane by hydrazinolysis (*18*). As shown in Fig. 2, binding of ^{125}I-anti-band 3 IgG to the band 3-Sepharose was significantly decreased when band 3 oligosaccharides were present. The inhibitory activity of the oligosaccharides was lowered by treatment with endo-β-galactosidase. Similarly, binding of radioactivity to the gel was decreased in the presence of lactoferrin, and the inhibitory activity of lactoferrin was lowered

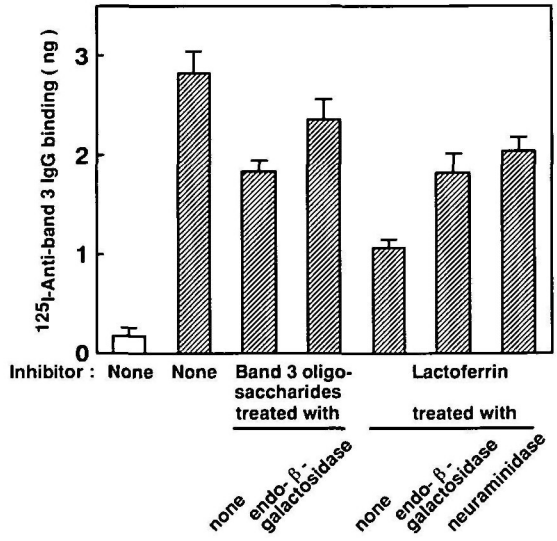

Fig. 2. **Effect of band 3 oligosaccharides and lactoferrin on binding of ^{125}I-anti-band 3 IgG to band 3-Sepharose gel.** A mixture of ^{125}I-anti-band 3 (0.15 μg/ml) with band 3 oligosaccharides, endo-β-galactosidase-treated oligosaccharides (750 μg/ml), or with lactoferrin, endo-β-galactosidase-treated or neuraminidase-treated lactorerrin (500 μg/ml), was preincubated at 4°C overnight, and centrifuged. Band 3-Sepharose gel (25 μl) was incubated with 200 μl of each supernatant at 4°C for 20 h. Control gel was incubated with the supernatant from ^{125}I-anti-band 3 alone. After washing of the gel with a buffer, the radioactivity of the control gel cake (open column) and the band 3-gel cake (hatched column) was counted. The data are the mean values±SD of triplicate experiments.

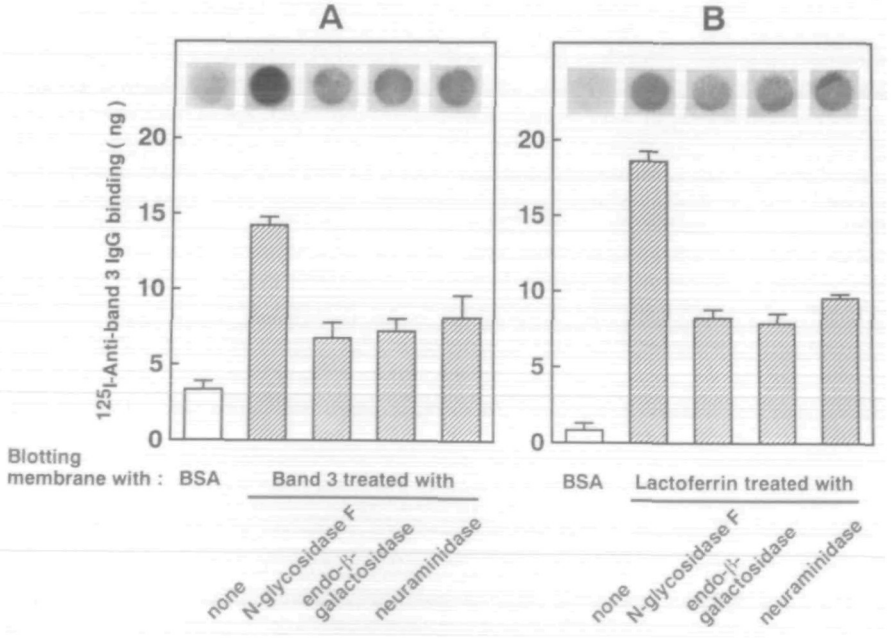

Fig. 1. **Binding of ^{125}I-anti-band 3 IgG to band 3-blotted membrane (A) and lactoferrin-blotted membrane (B).** A PVDF blotting membrane was adsorbed with 100 μg of BSA, band 3, *N*-glycosidase F-treated band 3, endo-β-galactosidase-treated band 3, or neuraminidase-treated band 3 (A); or adsorbed with 100 μg of BSA, lactoferrin, *N*-glycosidase-treated lactoferrin, endo-β-galactosidase-treated lactoferrin, or neuraminidase-treated lactoferrin (B). Each membrane was incubated in a solution of ^{125}I-anti-band-3 IgG (0.3 μg/ml) at 4°C for 4 h. Radioactivity of the membrane was measured by radioluminography. The columns express the mean values±SD of triplicate experiments. A representative radioactivity image of each sample is shown above the column. The amounts of band 3, *N*-glycosidase-F-treated band 3, endo-β-galactosidase-treated band 3, and neuraminidase-treated band 3 bound to the membrane were 57–58 μg, and those of lactoferrin, *N*-glycosidase F-treated lactoferrin, endo-β-galactosidase-treated lactoferrin, and neuraminidase-treated lactoferrin bound to the membrane were 62–64 μg, as assessed by using ^{125}I-labeled band 3 and lactoferrin, and those treated with the enzymes.

by treatment with endo-β-galactosidase or neuraminidase. Although the inhibitory effect of band 3 oligosaccharides and lactoferrin was partial at the concentrations used, the results are consistent with the results obtained in the blotting assays.

Release of radioactivity from the band 3-Sepharose gel, to which ^{125}I-anti-band 3 IgG was bound, upon glycosidase treatment was then examined (Fig. 3). Incubation of the gel with *N*-glycosidase F or endo-β-galactosidase resulted in a significant release of radioactivity from the gel, while little release of radioactivity was observed in control incubations without the enzymes. This result also supports the idea that binding of anti-band 3 IgG to band 3 is largely mediated by poly-*N*-acetyllactosaminyl sugar chains of the glycoprotein.

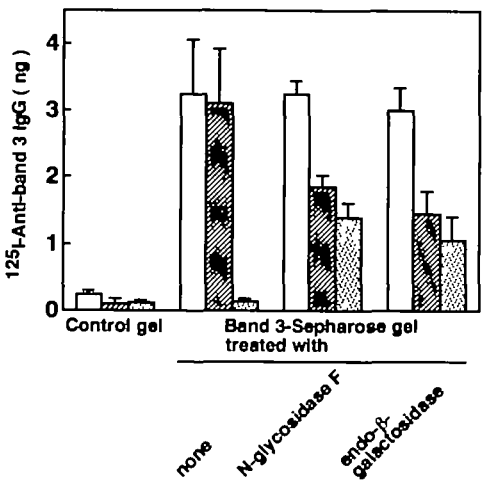

Fig. 3. **Release of radioactivity from band 3-Sepharose gel bound with ^{125}I-anti-band 3 IgG upon glycosidase treatment.** Band 3-Sepharose gel and control gel (25 μl) were incubated with 100 μl of ^{125}I-anti-band 3 (0.3 μg/ml) at 4°C for 20 h. Radioactivity of the gel cake (open column) was measured. The gel cake was incubated with 100 μl of a solution of *N*-glycosidase F at pH 7.4 (1 unit/ml), endo-β-galactosidase at pH 5.8 (100 milliunits/ml), or control buffers of pH 7.4 and 5.8 at 37°C for 4 h. Radioactivity of the gel cake (hatched column) and the medium (dotted column) was counted. Since no difference was seen in the results of the control incubations, only the result of the control incubation at pH 7.4 is shown (none). The data are the mean values±SD of triplicate experiments.

To assess the proportion of IgG having affinity for poly-*N*-acetyllactosamine-type sugar chains in the anti-band 3 IgG preparation, binding of ^{125}I-anti-band 3 IgG to a column of lactoferrin-Sepharose gel was measured. When ^{125}I-anti-band 3 IgG was loaded on the column of band 3-Sepharose gel as a control experiment, 79% of the radioactivity was retained on the column (Fig. 4A). When ^{125}I-anti-band 3 IgG was loaded on a lactoferrin-Sepharose column, 53% of the labeled anti-band 3 IgG was retained on the column (Fig. 4B). Thus, 67% (53/79×100%) of the active fraction of ^{125}I-anti-band 3 IgG bound to the lactoferrin-Sepharose column. It is therefore likely that about 67% of anti-band 3 IgG recognizes the sugar chains of band 3 and lactoferrin.

Binding Specificity of IgG Isolated by Affinity Chromatography Using a Lactoferrin Column—If anti-band 3 IgG contains antibody directed to poly-*N*-acetyllactosaminyl sugar chains, then it may be isolated from plasma by affinity chromatography using a column of lactoferrin-Sepharose gel. A small fraction of IgG of normal human plasma was isolated by use of the affinity column with a similar yield to that of anti-band 3 IgG. The isolated IgG, tentatively designated as anti-lactoferrin IgG, was labeled with ^{125}I, and its binding specificity was examined.

When ^{125}I-anti-lactoferrin IgG was loaded on a column of the lactoferrin-Sepharose gel, radioactivity retained on the column amounted to 82% (Fig. 5A). Similarly, 78% of the labeled anti-lactoferrin IgG was retained on the column of the band 3-Sepharose gel (Fig. 5B). Hence, anti-lactoferrin IgG recognizes band 3 glycoprotein. In the blotting assay, ^{125}I-anti-lactoferrin IgG effectively bound to the lactoferrin-blotted membrane as compared with the binding to the control BSA-blotted membrane, and binding of radioactivity to the membrane blotted with lactoferrin that had been pretreated with *N*-glycosidase F or endo-β-galactosidase was lower (Fig. 6A). ^{125}I-Anti-lactoferrin IgG also bound to the band 3-blotted membrane, and binding of radioactivity to the membrane blotted with band 3 that had been pretreated with *N*-glycosidase F or endo-β-galactosidase was nearly as low as that to the control BSA-blotted membrane (Fig. 6B). Thus, anti-lactoferrin IgG is directed to the poly-*N*-acetyllactosaminyl sugar chains of lactoferrin and band 3, and is a fraction of anti-band 3 IgG that recognizes the poly-*N*-acetyllactosamine structure. The results are consistent with the idea that anti-band 3 IgG

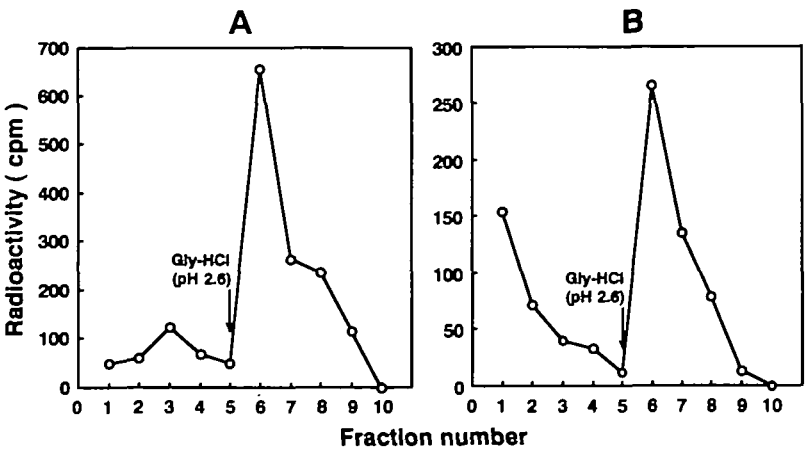

Fig. 4. **Binding of ^{125}I-anti-band 3 IgG to a band 3-Sepharose column (A) and a lactoferrin-Sepharose column (B).** A: ^{125}I-Anti-band 3 IgG (1,363 cpm) was applied to a column of band 3-Sepharose gel (0.5 ml). The column was eluted successively with 2 ml of DPBS(−) and 10 ml of 0.1 M glycine-HCl, pH 2.6. Each 0.5-ml fraction was collected and the radioactivity of the fraction was counted. B: ^{125}I-Anti-band 3 IgG (891 cpm) was applied to a column of lactoferrin-Sepharose gel (0.5 ml), and the column was treated similarly. Radioactivity recovered in the acidic eluates was counted. When ^{125}I-anti-band 3 IgG (2,460 cpm) was applied to a column of the control Sepharose gel (0.5 ml), very little radioactivity was retained on the column and recovered in the acidic eluates (less than 3%).

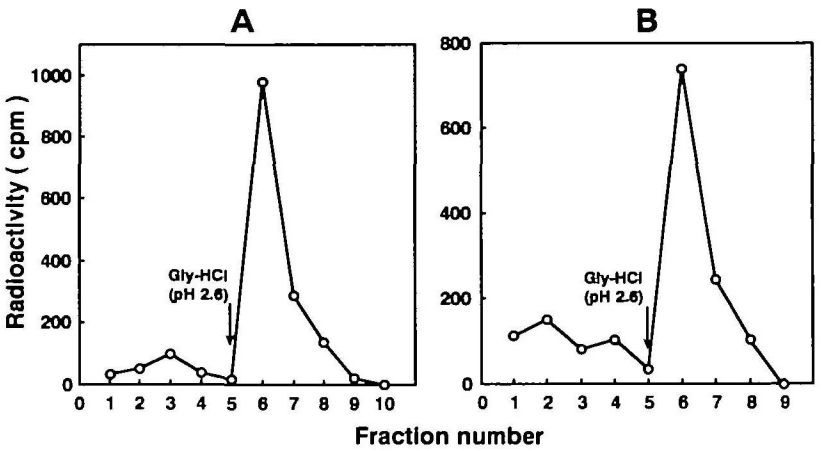

Fig. 5. **Binding of ^{125}I-anti-lactoferrin IgG to a lactoferrin-Sepharose column (A) and a band 3-Sepharose column (B).** A: ^{125}I-Anti-lactoferrin IgG (1,624 cpm) was applied to a column of lactoferrin-Sepharose gel (0.5 ml). The column was eluted successively with 2 ml of DPBS(−) and 10 ml of 0.1 M glycine-HCl, pH 2.6. Each 0.5-ml fraction was collected and the radioactivity of the fraction was counted. B: ^{125}I-Anti-lactoferrin IgG (1,600 cpm) was applied to a column of band 3-Sepharose gel (0.5 ml), and the column was treated similarly. Radioactivity recovered in the acidic eluates was counted. When ^{125}I-anti-lactoferrin IgG was applied to a column of the control Sepharose gel, no radioactivity was retained.

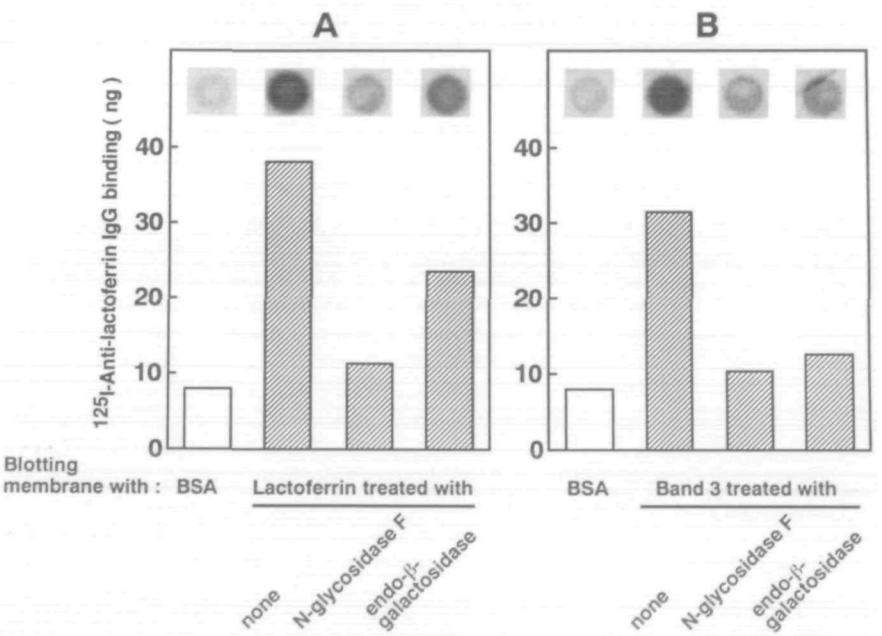

Fig. 6. **Binding of ^{125}I-anti-lactoferrin IgG to a lactoferrin-blotted membrane (A) and a band 3-blotted membrane (B).** A PVDF blotting membrane was adsorbed with 100 μg of BSA, lactoferrin, *N*-glycosidase F-treated lactoferrin or endo-β-galactosidase-treated lactoferrin (A); or BSA, band 3, *N*-glycosidase F-treated band 3 or endo-β-galactosidase-treated band 3 (B). Each membrane was incubated in a solution of ^{125}I-anti-lactoferrin IgG (2.5 μg/ml) at 4°C for 20 h. Radioactivity of the membrane was measured by radioluminography. The result shown is a representative one, and reproducible results were obtained in repeated experiments. A radioactivity image of each sample is shown above the column.

contains antibody to poly-*N*-acetyllactosamine structure.

Binding Specificity of IgG Isolated by Affinity Chromatography Using Band 3 Prepared by Another Method—Although all the above results are consistent with the idea that a significant part of anti-band 3 IgG is directed to the carbohydrate portion of band 3, the possibility still remains that we have missed a large amount of natural IgG antibody to the polypeptide portion of band 3 that might exist in normal plasma.

The major difference between the method of anti-band 3 IgG isolation employed in our laboratory and that of Lutz *et al.*, who reported, based on Western blotting analysis, that anti-band 3 IgG binds exclusively to band 3 polypeptide (*20, 21*), appears to be that we used band 3 purified from SDS-solubilized ghosts while Lutz *et al.* used band 3 from non-ionic Triton X-100-solubilized ghosts (*32*) for preparation of immobilized band 3 columns. Since SDS may destroy the polypeptide conformation of band 3 which might be recognized by some IgG population, we purified band 3 by solubilizing the ghosts using a nonionic detergent, $C_{12}E_8$, which was shown to have little effect on band 3 structure (*33*), and isolated anti-band 3 IgG using an affinity column coupled with this band 3 preparation. The binding specificity of this anti-band 3 preparation was compared with that of the routinely prepared anti-band 3 IgG.

The two band 3 preparations were treated with endo-β-galactosidase and *N*-glycosidase F, and subjected to SDS-polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue (CBB) staining or Western blotting and ^{125}I-anti-band 3 IgG binding (Fig. 7). Results obtained with band 3 purified from the SDS-solubilized ghosts and anti-band 3 IgG purified using this band 3 preparation as an adsorbent are shown in Fig. 7A. In Fig. 7A, the electrophoretic mobility of band 3 treated with *N*-glycosidase F appeared to be slightly greater than that of untreated band 3 (left, lanes a and c). This is consistent with the reported observation that enzymatic deglycosylation of band 3 results in an increase in the mobility in SDS-gel electrophoresis (*34*), suggesting that the band 3 sample was considerably deglycosylated. ^{125}I-anti-band 3 IgG bound to band 3 on the Western blot (right, lane a), but the binding

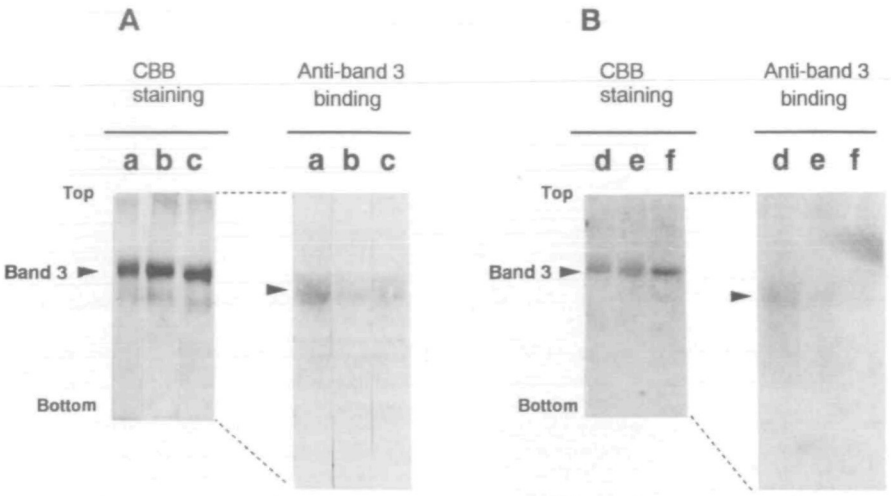

Fig. 7. Binding of ^{125}I-anti-band 3 IgG to Western blots of band 3. Four micrograms of band 3 (a and d), endo-β-galactosidase-treated band 3 (b and e), and *N*-glycosidase F-treated band 3 (c and f) were subjected to SDS-gel electrophoresis. The gels were stained with CBB (left), or subjected to Western blotting using a PVDF membrane followed by ^{125}I-anti-band 3 IgG binding assay as described in the legend to Fig. 1. A: Band 3 routinely purified by SDS-solubilization method (*22*), and anti-band 3 IgG isolated using this band 3 preparation as an adsorbent were used. B: Band 3 purified by the $C_{12}E_8$-solubilization method (*23*), and anti-band 3 IgG isolated using this band 3 preparation as an adsorbent were used.

was decreased when band 3 was treated with endo-β-galactosidase (right, lane b) or *N*-glycosidase F (right, lane c). The effect of the glycosidase treatment is consistent with that observed in direct blotting assay (Fig. 1A). Similar results were obtained in the experiment using band 3 purified from the $C_{12}E_8$-solubilized ghosts and anti-band 3 IgG purified using this band 3 preparation as an adsorbent (Fig. 7B): the electrophoretic mobility of *N*-glycosidase F-treated band 3 was greater than that of untreated band 3 (left, lanes d and f), and binding of ^{125}I-anti-band 3 IgG to band 3 on the Western blot (right, lane d) was decreased when band 3 was treated with endo-β-galactosidase (right, lane e) or *N*-glycosidase F (right, lane f).

These results indicate that there is no apparent difference in the antigenic properties between band 3 preparations purified from SDS-solubilized ghosts and nonionic $C_{12}E_8$-solubilized ghosts, and that at least a significant part of anti-band 3 IgG binds to poly-*N*-acetyllactosaminyl sugar chains of band 3.

DISCUSSION

It has been shown that naturally occurring antibodies play an important role in the phagocytic removal of senescent erythrocytes from the blood circulation (*3-5*). It seems most probable that the antibody is anti-band 3 IgG that binds to band 3-related antigens expressed on the cell surface. Kay *et al.* (*6*) demonstrated that band 3-related antigen is responsible for the autologous IgG binding by means of a phagocytosis inhibition study using IgG eluted from senescent erythrocytes by acid treatment, and using band 3 glycoprotein, and also by immunoautoradiography with anti-band 3. Kay postulated later that the antigens are polypeptide fragments of band 3 (*7*). Kay *et al.* (*8, 19*) and Lutz *et al.* (*20, 21*) have proposed that the antigenic sites on band 3 reside on its polypeptide moiety and not on saccharide residues. On the other hand, it has been shown that co-clustering of denatured hemoglobin with band 3 glycoprotein leads to the binding of anti-band 3 IgG in senescent erythrocytes (*11*). Low and coworkers (*35-37*) have shown that denatured hemoglobin exhibits high affinity binding to the cytoplasmic domain of band 3, which results in formation of a copolymer of hemoglobin and band 3, and they isolated the IgG-enriched complexes of proteins containing hemoglobin, band 3 and spectrin from senescent erythrocyte membrane (*12*). They suggested that the antigen is generated by clustering of band 3 glycoprotein in senescent erythrocytes.

Our series of experiments (*15-18*) has shown that oxidized and senescent erythrocytes generate band 3 antigen on the cell surface, to which anti-band 3 IgG binds, and the binding sites reside on sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3 glycoprotein. This proposal was compatible with the clustering theory (*10-14*), but inconsistent with the polypeptide fragment theory (*6-8, 19*). Lutz *et al.* (*21*) claimed that binding of anti-band 3 IgG to band 3 glycoprotein on the cell surface is not inhibited by lactoferrin, and they argued that the antigenic site resides on the polypeptide chains of band 3 glycoprotein.

The present investigation was carried out in order to demonstrate that anti-band 3 IgG can bind to band 3 glycoprotein through the sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3, by examining the direct interaction of anti-band 3 IgG with band 3 glycoprotein in cell-free systems. Human lactoferrin was used as another ligand for anti-band 3 IgG since it bears a sialylated di-*N*-acetyllactosaminyl sugar chain (*31*). In the blotting assay, anti-band 3 IgG effectively bound to lactoferrin, and binding of anti-band 3 IgG to band 3 and lactoferrin was decreased, by 50-70% of the original binding, after removal of their saccharide chains, after destruction of poly-*N*-acetyllactosamine structure, or after removal of sialyl residues (Fig. 1). In the gel binding assay, binding of anti-band 3 IgG to the band 3-Sepharose gel was decreased by band 3 oligosaccharides and lactoferrin, but their effect was lowered when their poly-*N*-acetyllactosamine structure was destroyed or sialyl residues were cleaved (Fig. 2). In addition, anti-band 3 IgG, bound to the band 3-Sepharose gel, was released by removal of the saccharide chains of the immobilized band 3 or by destruction of its poly-*N*-acetyllactosamine structure (Fig. 3). These observations were consistent with the fact that IgG reactive with both band 3 and lactoferrin, depending on their polylactosaminyl sugar chains, was also isolated by affinity chromatography using lactoferrin-Sepharose from normal human serum (Figs. 5 and 6). These results clearly demonstrate that anti-band 3 IgG primarily recognizes sialylated poly-*N*-acetyllacto-

saminyl sugar chains. Since lactoferrin contains only di-*N*-acetyllactosaminyl chains (*31*) and cleavage of the chains with endo-β-galactosidase destroyed their ligand activity (Figs. 1, 2, and 6), the antigenic determinant on band 3 and lactoferrin for the antibody should be NeuNAcα2-6(3)-Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-, which confirms our previous conclusion drawn from inhibition studies in anti-band 3 IgG binding to oxidatively damaged erythrocytes (*17*).

The present results do not agree with those reported by Lutz *et al.* (*21*). They totally excluded the possibility of the carbohydrate chains of band 3 as antigenic sites. This disagreement does not appear to be due to conformational or structural difference of band 3 used for isolation of anti-band 3 IgG since we have equally obtained anti-band 3 IgG with a carbohydrate-binding property whether band 3 denatured by SDS or band 3 minimally denatured by $C_{12}E_8$ was used as an immunoadsorbent for the antibody isolation (Fig. 7). Although our results indicate that the major antigenic sites for anti-band 3 IgG binding reside on the carbohydrate chains of band 3 glycoprotein, not all the band 3-binding activity of anti-band 3 IgG was explained by binding to the carbohydrate moiety, because endo-β-galactosidase or *N*-glycosidase F treatment of band 3 or band 3-Sepharose gel did not completely destroy the ^{125}I-anti-band 3 IgG binding to them (Figs. 1 and 3). This incompleteness may be due to incomplete digestion of the carbohydrate chains of band 3, but it is also possible that an antibody population that recognizes the polypeptide regions of band 3 exists and contributes to the anti-band 3 IgG-binding to the isolated band 3. Anti-band 3 IgG bound to lactoferrin-Sepharose in an amount 33% less than that to band 3-Sepharose (Fig. 4). Considering that anti-lactoferrin IgG bound to both types of gels equally well (Fig. 5), the fraction of anti-band 3 IgG which does not bind to lactoferrin-Sepharose may represent an anti-band 3 IgG population directed to the band 3 polypeptide. A good agreement between the proportion of anti-band 3 IgG not retained by lactoferrin-Sepharose (Fig. 4) and that of anti-band 3 IgG capable of binding to the glycosidase-treated band 3 (Fig. 1) supports this possibility.

The present results support our previous proposal that the antigenic sites on the surface of the oxidized or senescent erythrocytes are primarily sialylated poly-*N*-acetyllactosaminyl sugar chains of band 3 glycoprotein (*15-18*), although other glycoconjugates having polylactosamine structure such as band 4.5 glycoprotein and some glycolipids may also be involved. The present results are compatible with those of other groups, who suggested that the antigenic sites on oxidized or senescent erythrocytes are formed by clustering or aggregation of band 3 (*11-14, 38*). It is conceivable that the binding affinity of anti-band 3 IgG to band 3 on unoxidized or young erythrocytes is too low to allow stable binding, because band 3 is sparsely and evenly distributed. On the other hand, oxidation or aging of the cells may cause clustering or aggregation of band 3 molecules on the membrane, which would result in an increase in the local density of the saccharide chains of band 3, allowing high-affinity divalent binding of the antibody to the saccharides. Actually, band 3 of senescent erythrocytes has been shown to be oligomerized (*23*), and we have recently found that band 3 forms detergent-insoluble aggregates with other membrane proteins when erythrocytes are oxidized (Ando *et al.*, unpublished results).

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