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Binding of naturally occurring antibodies to oxidatively and nonoxidatively modified erythrocyte band 3

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Abstract

Both oxidative clustering (elicited by diamide treatment) and nonoxidative clustering (elicited by zinc/BS3 (bis[sulfosuccinimidyl]suberate) treatment) of erythrocyte integral membrane proteins induce binding of autologous antibodies with anti-band 3 specificity, followed by complement deposition and phagocytosis. Autologous antibodies eluted from nonoxidatively clustered erythrocytes bind to and stimulate phagocytosis of oxidatively damaged erythrocytes. Those eluted antibodies bind specifically to disulfide-crosslinked band 3 dimers generated by diamide treatment. Band 3 dimerization and antibody binding are abrogated by cleavage of band 3 cytoplasmic domain. Thus, disulfide-crosslinked band 3 dimers are the minimal band 3 aggregate with enhanced affinity for anti-band 3 antibodies. The eluted antibodies do not bind to band 3 dimers generated nonoxidatively by BS3 treatment but bind avidly to larger band 3 clusters generated nonoxidatively by zinc/BS3 treatment. Possibly, disulfide crosslinking of cytoplasmic domain cysteines induces reorientation of intramembrane domains as to expose putative anti-band 3 epitopes and allow bivalent binding of anti-band 3 antibodies. Extensive nonoxidative band 3 clustering appears to disrupt the native band 3 conformation and generate reoriented dimers which expose putative anti-band 3 epitopes in the proper distance and orientation as to allow bivalent antibody binding.

Key words: Band 3; Erythrocyte; Erythrocyte removal; Phagocytosis; Oxidative damage; Diamide

1. Introduction

Phagocytic removal of senescent, oxidatively damaged or pathological human erythrocyte appears to be mediated by deposition of autologous antibodies with anti-band 3 specificity [1–8]. Erythrocyte-bound antibodies activate alternate complement pathway and binding of complement components, notably C3b, eventually recognized by the complement receptor type one (CR1) on the phagocyte [6,9,10]. The intimate nature of senescence- or oxidation-mediated changes to band 3 which enhance binding of anti-band 3 antibodies, has not been defined as yet. Band 3 oxidative clustering, secondary or not to hemoglobin oxidation and hemoglobin binding to band 3 cytoplasmic domain has been considered to be the primary change leading to increased affinity and/or bivalent binding to anti-band 3 antibodies [4,5]. Stronger oxidative damage produced high-molecular weight band 3 aggregates, which were the selective binding site for anti-band 3 antibodies, complement and hemoglobin [11]. However, mild oxidative treatment of erythrocyte with diamide without evident formation of band 3 clusters also leads to anti-band 3/complement-mediated phagocytosis [6]. Nonoxidative band 3 clustering, induced by zinc, melittin or acridine orange generated high-molecular mass band 3 aggregates similar to the oxidatively generated aggregates in terms of selective binding of anti-band 3 antibodies, complement and hemoglobin, and phagocytosis induction [12,13]. No indications as to the structural changes responsible for enhanced IgG affinity of those aggregates are available.

Aim of this work is to characterize the minimal oxidative band 3 modifications capable to enhance binding of anti-band 3 antibodies and phagocytosis, and to find a common denominator between oxidative

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and nonoxidative band 3 modifications. We show here that the same autologous antibodies with anti-band 3 specificity eluted from nonoxidatively clustered erythrocyte also bind to disulfide-crosslinked band 3 dimers, where involved cysteines were localized on adjacent cytoplasmic domains. Thus, disulfide-crosslinked band 3 dimers constitute the minimal aggregate with enhanced affinity for anti-band 3 antibodies. While anti-band 3 antibodies have no affinity to covalent, nonoxidatively generated band 3 dimers, those antibodies avidly bind to larger nonoxidative band 3 clusters.

2. Materials and methods

2.1. Materials

Rabbit anti-human IgG antibodies conjugated to alkaline phosphatase, mouse anti-rabbit IgG antibodies conjugated to horseradish peroxidase and rabbit anti-human C3c antibodies (all affinity-purified polyclonals) were from Sigma. Antibodies to band 3 cytoplasmic domain raised in rabbits and affinity-purified with immobilized band 3 were a gift from P.S. Low (Purdue University). C12E8 (Nikkol) was from Nikkei Chem Co; BS3 (bis[sulfosuccinimidyl]suberate) was from Pierce; eosin 5-maleimide was from Molecular Probes; Protein A, Affigel-10 and Sepharose CL-6B were from Pharmacia; diamide, NEM (*N*-ethylmaleimide), PMSF (phenylmethylsulfonyl fluoride), Tween-20 were from Sigma. Sterile plastics were from Costar. All other reagents were purchased from common commercial sources.

2.2. Erythrocyte and membrane treatments

Freshly drawn, heparinized human blood was centrifuged at 3000 rpm for 7 min at room temperature. Plasma was saved and buffy coat removed by aspiration. Isolated erythrocytes were washed 3 × in Hepes-glucose (10 mM Hepes, 130 mM NaCl, 10 mM glucose (pH 7.4)). Washed erythrocytes were subjected to different treatments as specified. BS3 and zinc/BS3 treatment were performed as indicated [12]. Diamide (0.1–1.0 mM final concentration) was added to washed erythrocytes suspended at 10% hematocrit in Hepes-glucose at 4°C. After 30 min incubation on ice, cells were washed 3 × in Hepes-glucose. Labeling of band 3 by eosin 5-maleimide treatment was performed by incubating washed erythrocytes in Hepes-glucose containing 10 μM eosine 5-maleimide for 30 min at 4°C in the dark [14,15]. All procedures following eosine 5-maleimide labeling were also performed in the dark. Treatment with NEM was performed incubating washed erythrocytes in Hepes-glucose containing 20 mM NEM for 1 h at 20°C. Cells were then washed 3 × with Hepes-glucose. Erythrocyte-bound IgG were labeled incubating washed erythrocytes at 10% hematocrit in Hepes-glucose containing 2% bovine serum albumin (BSA) and supplemented with rabbit anti-human IgG conjugated to alkaline phosphatase (diluted 1:500) for 1 h at 4°C. Cells were then washed 5 × in Hepes-glucose/BSA. Membranes were prepared by hemolysis of washed erythrocytes in ice-cold 5 mM sodium phosphate buffer supplemented with 1 mM EDTA (pH 8.0), and washed 3 × in the same solution [16].

2.3. Assay of phagocytosis and erythrocyte-bound IgG

Control, diamide-treated, or zinc/BS3-treated erythrocytes were incubated in Hepes-glucose, in autologous serum, or in Hepes-glucose supplemented with autologous IgG eluted from zinc/BS3-treated erythrocytes (ElAb) (diluted 1:10) at 10% hematocrit for 60 min at 37°C. Cells were then washed 2 × in Hepes-glucose and used for phagocytosis assay or for measurement of bound IgG. Phagocytosis was assayed as described [17]. Briefly, erythrocytes were added to adherent human monocytes at a ratio of 200 erythrocytes per monocyte. After 1 h phagocytosis, noningested adherent cells were lysed and hemoglobin content in monocytes was quantified by measuring the heme peroxidase activity in a bioluminescence assay. Erythrocyte-bound IgG were measured after labeling erythrocytes with anti-human IgG conjugated to alkaline phosphatase as described above. Membranes were prepared, solubilized in PBS containing 0.5% Tween-20 (v/v) and alkaline phosphatase activity measured by visible spectrophotometry at 405 nm [18].

2.4. Isolation of autologous IgG eluted from zinc/BS3-treated erythrocytes (ElAb)

Washed zinc/BS3-treated erythrocytes were opsonized in fresh autologous serum at 33% hematocrit for 30 min at 37°C [12]. Cells were then washed 3 × in Hepes-glucose at 4°C and membranes prepared as described. One ml membranes were added to 1 ml 100 mM glycine (pH 3.0), incubated 5 min at 4°C under gentle shaking and centrifuged at 13000 rpm in an Eppendorf microcentrifuge. The supernatant was neutralized with 1 M imidazole (pH 7.8), and incubated with 0.2 ml Protein A beads overnight at 4°C. Beads were washed 5 × with PBS supplemented with 0.1% (v/v) Tween-20 and cell-associated autologous IgG (ElAb) eluted as described above. Protein concentration of eluted IgG was adjusted to 50 μg/ml with PBS. Immunoblots confirmed the presence of IgG and did not show reactivity with anti-C3 antibodies.

2.5. Immunoblotting of membrane proteins

Ten μg membrane proteins were separated on 5–15% polyacrylamide gel prepared according to Laemmli [19] and transferred to nitrocellulose as described [12]. After blocking for 1 h in PBS containing 5% (w/v) BSA, first antibodies were added to PBS containing 1% (w/v) BSA. Anti-band 3 was diluted 1:1000 in PBS and incubated for 1 h at room temperature. ElAb were diluted 1:500 in PBS and incubated for 12 h at room temperature. After washing the nitrocellulose by suction, second antibodies were diluted 1:1000 in PBS and incubated for 1 h at room temperature. The following second antibodies were used: anti-rabbit IgG conjugated to horseradish peroxidase for visualizing anti-band 3 antibodies; anti-human IgG conjugated to alkaline phosphatase for visualizing ElAb. Blots were developed with 4-chloronaphthol for peroxidase or with nitro blue tetrazolium/bromochlorophosphate for alkaline phosphatase [18].

2.6. Localization of cell-associated IgG and band 3 in nondenaturing detergent-extracted, labeled membrane proteins separated on Sepharose CL-6B

Membranes were isolated from erythrocytes treated with 0.5 mM diamide or from untreated control erythrocytes, labeled with rabbit anti-human IgG conjugated to alkaline phosphatase and eosin 5-maleimide (see above) or left unmodified. One ml membranes was prepared as described and solubilized in 2 ml of extraction buffer (10 mM Hepes, 130 mM NaCl, 10 mM *N*-ethylmaleimide, 1 mM EDTA, 1 mM PMSF, 1.5% C12E8 (pH 7.4)). After addition of 2 volumes extraction buffer, membranes were gently shaken for 20 min at 37°C and pelletted at 13000 rpm for 1 min in an Eppendorf microcentrifuge. The supernatant was applied to a 100 × 1.7 cm column filled with Sepharose CL-6B equilibrated with a solution containing 10 mM Hepes, 50 mM NaCl, 0.1% C12E8 (pH 7.4), at a flow rate of 0.9 ml/min. Constant flow was maintained using a HPLC pump. The effluent was collected in 4.5 ml fractions. Fractions were analyzed for protein (by absorbance at 280 nm), band 3 (by fluorescence of the eosin 5-maleimide/band 3 adduct), and IgG (by absorbance at 405 nm of the alkaline phosphatase activity). To remove IgG from the band 3 containing fractions, 1 ml from each fraction was incubated with 50 μl Sepharose CL-6B/Protein A under gentle shaking overnight at 4°C. Incubation was stopped by centrifugation. Samples were concentrated in presence of 1% SDS by vacuum centrifugation to equalize band 3 concentration, as estimated by eosin 5-maleimide fluorescence in the peaks, electrophoresed and blotted as described. To remove IgG from the band 3 containing fractions, 1 ml of each fraction was incubated with 50 μl Sepharose CL-6B/Protein A under gentle shaking overnight at 4°C. Incubation was stopped by centrifugation.

2.7. Separation and identification of disulfide-crosslinked dimers of band 3 cytoplasmic domains ('80-kDa fragments') in diamide-treated erythrocytes

Dimers of band 3 cytoplasmic domains (80-kDa molecular mass, '80-kDa fragments') are masked by monomeric band 3. To lower this interference, staining intensity of monomeric band 3 was reduced by proteolytic splitting of band 3 into 35- and 60-kDa fragments [20]. Erythrocytes were incubated at 10% hematocrit in Hepes-glucose containing 1 mg/ml (w/v) chymotrypsin for 1 h at 37°C. Cells were then washed 5 × with

Table 1
Binding of IgG eluted from zinc/BS3-treated erythrocytes, and induction of phagocytosis in variously treated erythrocytes

Erythrocyte treatment	Erythrocytes incubated with						
	PBS	*P*	serum	*P*	ElAb	*P*	
Control	1.0 ± 0.2		1.0 ± 0.3		0.9 ± 0.2		A
	–		1.0 ± 0.4		1.1 ± 0.5		B
Diamide (0.1 mM)	1.1 ± 0.1	NS	2.7 ± 0.4	< 0.01	4.6 ± 0.5	< 0.01	A
	–		0.9 ± 0.2	NS	4.2 ± 2.3	0.019	B
Diamide (0.5 mM)	1.4 ± 0.2	0.030	3.0 ± 0.3	< 0.01	5.3 ± 0.7	< 0.01	A
	–		2.0 ± 0.9	0.053	15.1 ± 4.8	< 0.01	B
BS3 (1 mM)	1.1 ± 0.1	NS	1.4 ± 0.3	0.068	1.5 ± 0.4	0.017	A
	–		1.3 ± 0.3	NS	1.3 ± 0.4	NS	B
Zinc/BS3	1.8 ± 0.6	0.045	5.8 ± 1.1	< 0.01	8.3 ± 2.2	< 0.01	A
	–		29.5 ± 7.6	< 0.01	35.1 ± 8.7	< 0.01	B

After the indicated treatments, erythrocytes were incubated with PBS, fresh autologous serum or IgG eluted from zinc/BS3-treated erythrocytes (ElAb) as detailed in Materials and methods. Values represent relative phagocytosis in treated vs. untreated control erythrocytes incubated in PBS (A), or relative ElAb binding in treated vs. untreated control erythrocytes incubated in serum (B). Values are means ± S.D. of 4 or 5 separate experiments. Phagocytosis of control erythrocytes was 0.6 ± 0.2 erythrocytes per monocyte ($n = 5$). Statistical analysis (t-test for paired samples) was performed on comparisons between treated and nontreated control erythrocytes. Phagocytosis and binding of ElAb were assayed as detailed in Materials and methods. NS, not significant.

Hepes-glucose. Membranes were prepared and immediately incubated in 10 volumes of ice-cold PBS containing 1 μg/ml (w/v) trypsin for 1 h on ice. Incubation was stopped with 1 mM PMSF, membranes separated by centrifugation and sample buffer was added to the pellet. Membranes were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-band 3 antibodies as described above.

2.8. Band 3 depletion by immunoprecipitation

1 mg anti-band 3 antibody was bound to Affigel-10. 100 μl membranes were solubilized with extraction buffer as for gelfiltration (see above) and added to the matrix-bound anti-band 3 antibody. Solubilized membranes were then incubated under gentle shaking overnight at 4°C. The matrix was separated by centrifugation, the supernatant added to 5% SDS and used for immunoblotting.

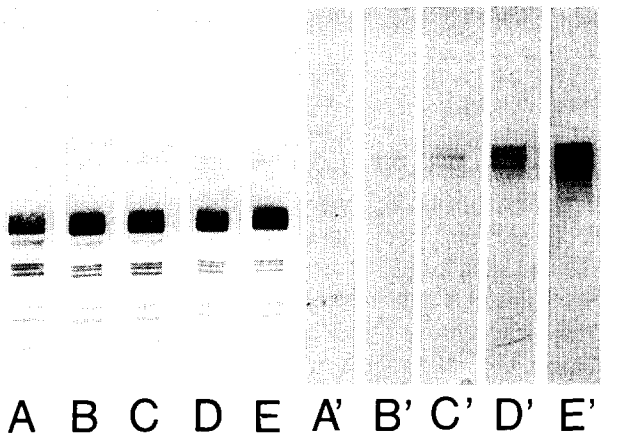

Fig. 1. Immunoblots of membrane proteins extracted from diamide-treated erythrocytes with anti-band 3 antibodies or IgG eluted from zinc/BS3-treated erythrocytes. Membranes were isolated from erythrocytes treated with 0 (A), 0.02 (B), 0.1 (C), 0.5 (D), 1.0 (E) mM diamide. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-band 3 antibodies (A–E) or with IgG eluted from zinc/BS3-treated erythrocytes (A'–E').

3. Results

3.1. IgG eluted from zinc/BS3-treated erythrocytes (ElAb) bind to diamide-treated erythrocytes and induce their phagocytosis

In previous work, zinc treatment was shown to induce nonoxidative clustering of band 3 [12]. The clustered distribution was stabilized by the impermeable crosslinker BS3 (zinc/BS3 treatment), allowing removal of the zinc without loss of the induced affinity for autologous IgG. IgG eluted from zinc/BS3-treated erythrocytes (ElAb) were utilized here to opsonize diamide-treated erythrocytes. As shown in Table 1, ElAb bind to diamide-treated erythrocyte and induce their phagocytosis proportionally to the diamide concentration used. As expected, treatment with zinc/BS3 strongly enhances phagocytosis and ElAb binding. ElAb do not bind to control erythrocytes or to erythrocytes treated with BS3, do not affect their phagocytic recognition, and are more effective phagocytic inducers than fresh autologous serum.

3.2. IgG eluted from zinc/BS3-treated erythrocytes (ElAb) bind to disulfide-crosslinked band 3 oligomers

In order to localize and characterize the membrane modifications that enhance ElAb binding to diamide-treated erythrocytes, the ability of ElAb to bind to membrane proteins extracted from diamide-treated erythrocytes separated by SDS gel electrophoresis and blotted onto nitrocellulose was tested. As shown in Fig. 1, ElAb bind to an approx. 200-kDa band which reacts positive with anti-band 3 antibodies. The 200-kDa band, which is present in diamide-treated erythrocyte but not in control erythrocyte, could represent disulfide-crosslinked band 3 dimers. To test this hypothesis, accessible band 3 -SH groups were protected by alkylation with NEM performed in situ before the diamide treatment. Secondly, mercaptoethanol or dithioerytritol were added to the solubilized protein samples before SDS-polyacrylamide gel electrophoresis. Both treatments resulted in the disappearance of the ElAb-reactive band (not shown). It should be noted that the 200-kDa band observed in immunoblots is less diffuse than the region corresponding to monomeric band 3. Scanning of the Coomassie blue-stained monomeric band 3 region shows that diamide treatment markedly decreased the faster migrating component of band 3 (not shown). This result could indicate preferential disulfide bond formation between less glycosylated band 3 components.

To provide additional evidence that the ElAb-reactive protein is band 3 dimer/oligomer, membranes were solubilized in nonionic detergent and band 3 removed by immunoprecipitation with anti-band 3 antibodies. Those band 3-depleted membranes had markedly reduced reactivity to anti-band 3 antibodies and ElAb (not shown). Each band 3 cytoplasmic domain contains 2 cysteines (cys-201, cys-317). Those cysteines are localized at the interface of adjoining band 3 monomers and reportedly mediate intermolecular crosslinking following oxidative treatments [21]. Plausibly, cys-201 and cys-317 also mediate the band 3 dimerization described above. As shown in Fig. 2, this assumption is strengthened by the presence of disulfide-linked dimers of band 3 cytoplasmic domains ('80-kDa fragments') isolated from membranes of diamide-treated erythrocytes. Moreover, trypsin cleavage of

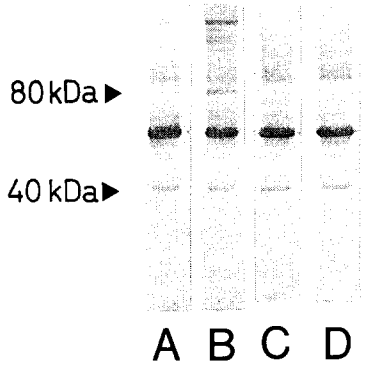

Fig. 2. Presence of disulfide-crosslinked dimers of band 3 cytoplasmic domains ('80-kDa fragments') in membranes of diamide-treated erythrocytes. Membranes were isolated from erythrocytes treated with 0.5 mM diamide. After cleavage of cytoplasmic domains, membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-band 3 cytoplasmic domain antibodies. Nonreduced (A) or reduced (1% mercaptoethanol) (C) membranes from control erythrocytes; nonreduced (B) or reduced (1% mercaptoethanol) (D) membranes from diamide-treated erythrocytes.

band 3 cytoplasmic domain abolished ElAb reactive band in immunoblots (not shown).

3.3. Nonoxidatively generated band 3 dimers have no affinity to ElAb

BS3, an impermeant bifunctional reagent, dimerizes band 3 by crosslinking two lysine residues localized in the intramembrane domains of two adjacent band 3 monomers (lys-lys dimers) [22,23]. As indicated in Fig. 3, those BS3-generated lys-lys dimers have no affinity to ElAb. When BS3 is added to zinc-treated erythrocyte, high-molecular mass aggregates are formed. These aggregates, which contain lys-lys linked dimers and no disulfide-crosslinked dimers, bind large amounts of ElAb (Fig. 3). This result could indicate that BS3-generated lys-lys dimers become recognizable by ElAb only if they cluster in large aggregates. As indications that ElAb binding to blotted membrane proteins could reflect ElAb binding to erythrocyte surface, it is possible to note that treatments (zinc/BS3, diamide) which stimulate ElAb binding to erythrocytes and erythrocyte phagocytosis also cause membrane alterations recognized by ElAb binding in immunoblots.

3.4. Disulfide-crosslinked band 3 dimers form high molecular weight aggregates which bind autologous IgG and complement

Similarly to zinc/BS3 treatment, also diamide treatment induces formation of high-molecular mass aggregates containing band 3, autologous IgG and complement. Membrane proteins were extracted by nondenaturing detergent, solubilized and separated on

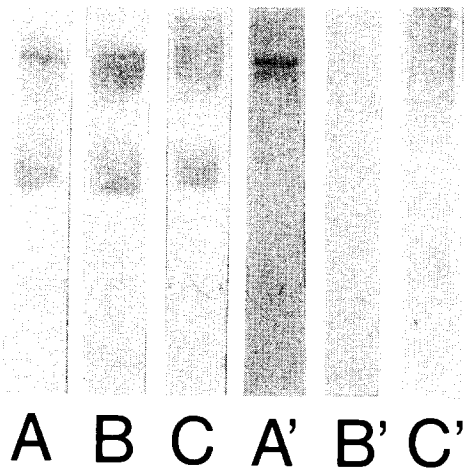

Fig. 3. Immunoblots of membrane proteins extracted from oxidatively and nonoxidatively treated erythrocytes with anti-band 3 antibodies or IgG eluted from zinc/BS3-treated erythrocytes. Membrane were isolated from erythrocytes treated with 0.5 mM diamide (A,A'), BS3 (B,B') or zinc/BS3 (C,C'). Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-band 3 antibodies (A,B,C) or with IgG eluted from zinc/BS3-treated erythrocytes (A',B',C').

Sepharose CL-6B, and fractions analyzed for protein, band 3 and IgG content. As shown in Fig. 4, diamide treatment leads to formation of > 6000 kDa molecular mass aggregates (Fig. 4, peak b), which are eluted in the column void volume. As expected, analysis of the material collected in peak a and peak b by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-band 3 antibodies or with ElAb (Fig. 5), reveals that peak b is enriched with disulfide-cross-

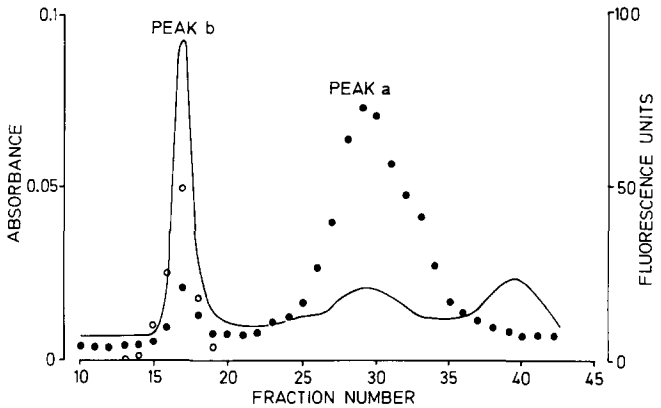

Fig 4. Gelfiltration chromatography of nondenaturing detergent extract of diamide-treated erythrocytes opsonized with IgG eluted from zinc/BS3-treated erythrocytes. Membranes were isolated from erythrocytes treated with 0.5 mM diamide, labeled with rabbit anti-human IgG conjugated to alkaline phosphatase, with eosin 5-maleimide or left unmodified. Membranes were extracted with 1.5% C12E8 and the supernatant applied to a 100×1.7 cm Sepharose CL-6B column. Fractions were collected and analyzed for protein (absorbance at 280 nm (———)), band 3 (fluorescence of the eosin 5-maleimide adduct (●)), and IgG content (absorbance at 405 nm of the alkaline phosphatase activity (○)).

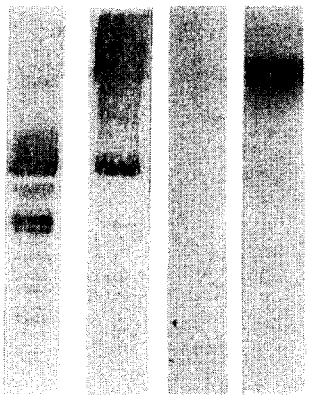

Fig 5. Immunoblots of nondenaturing detergent extracts corresponding to peak a and peak b of Fig. 4. Fractions corresponding to peak a (lanes A and C) and peak b (lanes B and D) of Fig. 4 were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-band 3 antibodies (A,B) or with IgG eluted from zinc/BS3-treated erythrocytes (C,D).

linked band 3 dimers, which copurify with IgG. Apparently, covalent band 3 dimers, irrespective of the type of elemental crosslink, de-stabilize the homogeneous distribution of band 3 and induce the formation of large aggregates. Peak b in control erythrocyte does not contain any detectable band 3 or autologous IgG. To disclose whether co-purification of IgG and aggregated band 3 was due or not to mutual binding, the material contained in peak b was incubated in the presence of matrix-bound protein A and band 3-associated eosin 5-maleimide fluorescence was measured following IgG immunoprecipitation. A decrease in the eosin 5-maleimide fluorescence of peak b by $32 \pm 14\%$ ($n = 4$) was observed following protein A incubation, indicating that band 3 and IgG were bound to each other. No change in band 3 content was observed upon incubation of peak a with protein A.

4. Discussion

Previous work has shown that oxidative damage induces the formation of large band 3 aggregates in erythrocyte membrane [4–6,10,11]. Those aggregates bind autologous IgG with anti-band 3 specificity and enhance phagocytic recognition [6,10,11]. Alternatively, also nonoxidatively generated band 3 aggregates bind large numbers of autologous IgG and induce phagocytosis [12]. Oxidative and nonoxidative treatments act differently on band 3. Oxidative agents, such as diamide or copper/*o*-phenanthroline, dimerize band 3 by oxidatively crosslinking cysteines localized in the cytoplasmic domains [24]. Nonoxidative agents, such as BS3, produce band 3 dimers by crosslinking lysine residues localized in the transmembrane domains [23]. Eventually, both oxidative and nonoxidative treatments ensue into the formation of large band 3 aggregates or clusters. (It is not obvious that differently generated band 3 modifications may be recognized by the same antibody). Here we provide evidence that the same autologous IgG eluted from nonoxidatively treated erythrocyte recognize disulfide-crosslinked band 3 dimers, and oxidatively or nonoxidatively generated large band 3 aggregates, but do not recognize nonoxidatively generated, covalently linked band 3 dimers. Thus, disulfide-crosslinked band 3 dimers represent the elemental covalent band 3 modification capable to dramatically enhance binding of anti-band 3 antibodies. Band 3 has the capacity to transduce specific intracellular modifications to the external side of the membrane, and make the erythrocyte recognizable by macrophages. It has been shown [25] that bivalent binding of anti-band 3 antibodies (as of any antibody) exponentially raises binding affinity. Thus, distance and relative orientation of two putative epitopes localized on two band 3 monomers appear to be crucial to allow bivalent binding of the antibody. Native band 3 in nonsenescent erythrocyte is physiologically present as a mixture of dimers and tetramers [26,27]. Evidently, since nonsenescent erythrocyte do not bind anti-band 3 antibodies and are not recognized by phagocytes, putative band 3 epitopes are not available for bivalent antibody binding in native dimers. We hypothesize that disulfide-crosslinking of cytoplasmic domain cysteines by oxidant treatment induces reorientation of the intramembrane domains in such a way as to bring putative anti-band 3 epitopes into the proper position to allow bivalent antibody binding. Apparently, extensive nonoxidative band 3 clustering disrupts the native band 3 conformation and generates re-oriented dimers with properly positioned putative anti-band 3 binding sites as to allow bivalent antibody binding. Detailed mechanistic explanation on how those large aggregates are formed in oxidatively or nonoxidatively modified erythrocyte is not available yet. However, literature data indicate that oxidation of cysteines of band 3 cytoplasmic domain decreases the affinity of band 3 for ankyrin [24]. Looser connection to cytoskeleton may increase band 3 lateral mobility [27,28] and facilitate intermolecular interactions. In addition, modifications of band 3 quaternary conformation may expose hydrophobic regions normally located at the interface of band 3 dimers or tetramers. Also, covalently modified band 3 dimers were found to undergo conformational changes leading to SDS-resistant noncovalent dimer/dimer associations [29]. Our observation that apparently underglycosylated band 3 has a higher tendency to cluster may also derive from its lower electrostatic repulsion.

In conclusion, our results indicate that band 3 is recognized by specific autologous antibodies if (a)

(quaternary) structure is modified by oxidative cross-linking of cytoplasmic domains, with ensuing reorientation of band 3 within the dimers and (b) it is nonoxidatively clustered in large aggregates where band 3 monomers are randomly oriented in the plane of the membrane. In the latter case, the chance of two contiguous molecules having a proper reciprocal orientation and distance to allow bivalent antibody binding should increase with the dimension of the aggregate.

Since non-erythroid band 3 isoforms are present in the cytoplasmic membranes of many cell types and tissues [30,31], and oxidative insult is a common element to many kinds of cellular damage [32], the present results may have a more general significance in non-self generation and recognition/removal by phagocytic cells.

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