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High-Pressure–Induced Hemolysis in Papain-Digested Human Erythrocytes Is Suppressed by Cross-Linking of Band 3 *via* Anti–Band 3 Antibodies

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Upon exposure of human erythrocytes to a high pressure of 200 MPa, both hemolysis and vesiculation occur. The hemolysis of erythrocytes at 200 MPa was enhanced by removal of sialic acids from the membrane surface with papain. However, such enhancement was suppressed by cross-linking of band 3 *via* an anti–band 3 antibody (AB3A), which recognizes the exofacial domain of band 3, or by clustering of band 3 *via* Zn^{2+}. On the other hand, the size of high-pressure–induced vesicles increased from 423 to 525 nm in diameter upon exposure to papain of erythrocytes, but decreased to 444 nm with following treatment with AB3A. In these vesicles, the content of spectrin relative to band 3 was almost the same. Furthermore, the band 3–cytoskeleton interactions in erythrocyte membranes remained unaltered upon treatment with papain and AB3A. Flow cytometric analysis demonstrated that papain-pretreated erythrocytes mainly produce open ghosts at 200 MPa and that the production of such open ghosts is suppressed by AB3A. Thus, upon removal of negative charges from the membrane surface, open ghosts are readily produced due to the release of larger vesicles under pressure. Upon cross-linking of band 3 *via* AB3A, however, the release of smaller vesicles at 200 MPa is facilitated so that high-pressure–induced hemolysis is suppressed.

Key words: band 3, erythrocyte, flow cytometry, hemolysis, high pressure, vesiculation.

Abbreviations: AB3A, anti–band 3 antibody; BSA, bovine serum albumin; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Ig G, immunoglobulin G; PBS, phosphate-buffered saline, 10 mM sodium phosphate, 150 mM NaCl, pH 7.4; PB, 0.1 M sodium phosphate buffer, pH 7.4; PVP, polyvinylpyrrolidone.

Band 3 is an anion transport protein comprising 911 amino acid residues (*1*). Each erythrocyte contains about 10^6 band 3 molecules (*2*). In the erythrocyte membrane, band 3 molecules exist as a dimers and higher oligomers (*3*). Band 3 dimers are ankyrin-free, whereas band 3 tetramers account for about 30% of total band 3 and are associated with β-subunits of spectrin *via* ankyrin (*4*). Namely, the cytoplasmic domain of band 3 links the spectrin-based cytoskeleton to the bilayer (*5*). Defects in such vertical protein-protein interactions result in membrane instability and reduced deformability characterized by lipid bilayer destabilization (*6*). Thus, vesicles are released from the membrane surface of such erythrocytes (*7*).

Each membrane protein in erythrocytes has been investigated in detail from biochemical and biophysical points of view. However, information about protein–protein interactions and protein–lipid ones is lacking for the whole cell membrane. Analysis of the hemolysis and vesiculation of human erythrocytes under high pressure is expected to provide useful information about these interactions. High-pressure–induced hemolysis is enhanced upon digestion with proteinases such as trypsin and chymotrypsin (*8*), whereas is suppressed upon denaturation of spectrin at 49°C (*9*), and upon cross-linking of membrane proteins by diamide (*10*) or carbodiimide (*11*). Additionally, high-pressure–induced hemolysis is affected by anion transport inhibitors such as diisothiocyanostilbene disulfonate (*12*, *13*), suggesting that band 3 plays an important role in high-pressure–induced hemolysis. On the other hand, pressure vesicles induced from intact erythrocytes or 49°C-pretreated ones contain spectrin in addition to band 3 (*9*, *14*). In these smaller vesicles, the content of spectrin relative to band 3 is decreased (*9*, *14*). Interestingly, protein 4.1-rich vesicles are released under pressure from diamide-treated erythrocytes (*15*). Even when erythrocytes are exposed to severe conditions such as a pressure of 200 MPa, the response of the erythrocyte membrane to high pressure provides unique information about the interactions among membrane components.

To elucidate the contribution of band 3 to the membrane stability under pressure, we have decided to use an anti–band 3 antibody (AB3A), which recognizes the exofacial domain of band 3. The effects of band 3 cross-linking by AB3A on membrane stability are confirmed by using Fab fragments of AB3A. In general, Fab fragments are prepared by papain digestion of immunoglobulin G (Ig G) (*16*). Like chymotrypsin, papain is able to cleave the membrane domain of band 3 and the exoplasmic

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domain, which bears negative charges due to attached sialic acids, of glycophorins. Under mild conditions, the site of cleavage of band 3 by papain is close to that for chymotrypsin (*17*). Band 3 in intact erythrocytes is resistant to trypsin (*18*). On the other hand, the hemolytic properties at 200 MPa of erythrocytes treated with neuraminidase, trypsin, and chymotrypsin, respectively, are similar to each other (*8*), indicating that cleavage of the band 3 membrane domain is not so critical. Thus, papain has been used to prepare the Fab fragments of AB3A and to remove negative charges on the membrane surface. In the present work, we found that the hemolysis and vesiculation of papain-digested erythrocytes at 200 MPa are significantly affected by cross-linking of band 3 *via* AB3A.

MATERIALS AND METHODS

Materials—Compounds were obtained from the following sources: bovine serum albumin (BSA) fraction V, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and zinc chloride, Wako Chemicals; 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS), Tokyo Kasei; horseradish peroxidase–conjugated goat anti–rabbit Ig G and *N*-[*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl]- agmatine (E-64), Boehringer Mannheim; eosin-5-maleimide, Molecular Probes; FITC-conjugated goat anti-rabbit IgG $F(ab')_2$ fragment, Jackson ImmunoResearch; octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$), Nikko Chemicals; papain from papaya latex and polyvinylpyrrolidone (PVP), Sigma. All other chemicals were of reagent grade.

Preparation of AB3A and Its Fab Fragments—Band 3 was purified from human erythrocyte membranes, according to the method of Casey *et al*. (*19*). AB3A was prepared from rabbits. Band 3 was detected as a single band on Western blot analysis using AB3A as a primary antibody and horseradish peroxidase–conjugated goat anti–rabbit IgG as a secondary antibody. Fab fragments of AB3A were prepared as follows. To activate papain, papain (0.5 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4 (PB), containing 10 mM cysteine and 2 mM EDTA was incubated for 30 min at 37°C. One volume of antiserum was mixed with three volumes of activated papain solution, followed by incubation for 2 h at 37°C.

Erythrocyte Treatments—Human blood was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged at 1,000 × *g* for 10 min at 4°C. The plasma and buffy coat were carefully removed. The erythrocytes were washed three times with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.4). For papain treatment, intact erythrocytes (20% hematocrit) in PB were incubated with activated papain (0.5 mg/ml) for 1 h at 4°C. After incubation, the erythrocytes were washed three times in PBS. The proportion of sialic acids removed from the erythrocyte membrane was estimated as described previously (*8*). Intact erythrocytes or papain-digested ones were suspended at 3% hematocrit in antiserum diluted 4-fold with PB or in an antiserum solution containing Fab fragments, and then incubated for 1 h at 4°C. After incubation, the erythrocytes were washed three times in PBS, and then used for the pressure experiments, and the detection of antibody binding and cell agglutination. For high-pressure–induced hemolysis, the following samples were used in addition to the samples treated with papain and antiserum. Namely, papain-digested erythrocytes were suspended at a 20% hematocrit in HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 2.5 mM $ZnCl_2$, and then incubated for 15 min at 37°C . After incubation, the cell suspension was diluted with HEPES buffer containing Zn^{2+} and exposed to a pressure of 200 MPa. To remove Zn^{2+} bound to the erythrocytes, the cells were washed with PB. The Zn^{2+}-free cells in HEPES buffer were used for pressure experiments. Furthermore, intact erythrocytes were suspended at 2% hematocrit in PBS containing PVP (50 mg/ml) or BSA (50 mg/ml). Three volumes of such cell suspensions were mixed with one volume of antiserum or buffer. The cell suspensions were incubated for 30 min at 37°C. Such erythrocytes were washed two times in PBS prior to exposure to high pressure. To detect the binding of antibodies to the membrane, the erythrocytes were incubated with FITC-conjugated goat anti–rabbit IgG fragments [$F(ab')_2$] for 30 min at 37°C, washed in PBS, and then analyzed by flow cytometry. Additionally, the agglutination of erythrocytes was observed under a light microscope (Olympus, model IX 71).

High Pressure Treatment—Exposure to high pressure of erythrocytes was carried out as previously described (*20*). Briefly, erythrocyte suspensions (0.3 or 4% hematocrit) in PBS or HEPES (only in the case of Zn^{2+}) were subjected to a pressure of 200 MPa for 30 min at 37°C. After decompression, the suspensions were used for flow cytometry or were centrifuged for 10 min at 750 × *g* and 30°C to prepare vesicles and to measure hemolysis. For hemolysis, the absorbance of the supernatant was measured at 542 nm. One hundred percent hemolysis was achieved by adding a 0.3% volume of 5% Triton X-100 to the suspension.

Vesicles—Erythrocyte suspensions subjected to a pressure of 200 MPa for 30 min at 37°C were centrifuged at 750 × *g* for 10 min at 30°C. The supernatants were filtered using a Millipore filter (pore size, 3 μm). For SDS-PAGE analysis of membrane proteins in vesicles, the filtrates were centrifuged at 20,000 × *g* for 30 min at 4°C. The membrane protein composition of pellets was analyzed by SDS-PAGE. The gels were stained with Coomassie Blue. The stained bands were analyzed with an Advantec DM-303 scanning densitometer. For vesicle size determination, the filtrates containing AB3A were incubated with papain (0.5 mg/ml) for 30 min at 37°C. The filtrates were used to determine the vesicle size by the light scattering method (*9*).

Protein Extraction with $C_{12}E_8$—Intact erythrocytes were suspended at 20% hematocrit in PBS containing 0.5 mM DNDS and incubated for 10 min at 37°C prior to the addition of eosin-5-maleimide (0.1 mg/ml). The cell suspensions containing both DNDS and eosin-5-maleimide were incubated for 15 min at room temperature (26°C). After incubation, the erythrocytes were washed three times with PBS, once with PBS containing 0.5% (w/v) BSA, and then three times with PBS. Eosin-5-maleimide–labeled erythrocytes (20% hematocrit) in PB were incubated with activated papain (0.5 mg/ml) for 1 h at 4°C. The digested erythrocytes were washed three times with PBS, once with PBS containing 0.5% BSA, and then three times with PBS containing E-64 (10 μM). Ghosts were prepared

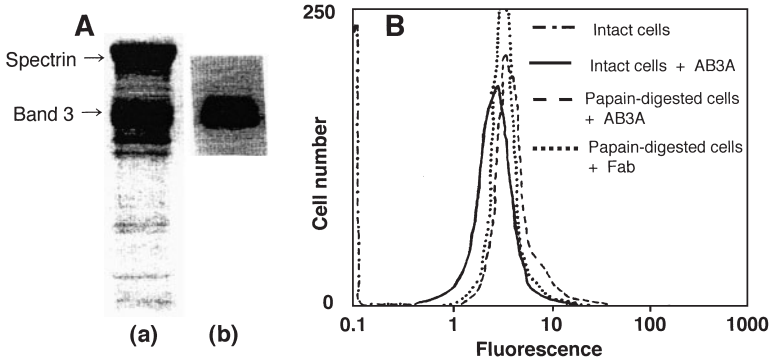


Fig. 1. **Detection of band 3 using AB3A, and binding to band 3 of AB3A and its Fab fragments.** (A) Band 3 detection by Western blotting using AB3A as a primary antibody. Membrane proteins in ghosts were separated by SDS-PAGE, and then detected by Coomassie Blue staining (lane a), or with a primary antibody (AB3A) and a secondary antibody (lane b). (B) Binding of AB3A or its Fab fragments to band 3 in the erythrocyte membrane was examined by flow cytometry. Primary antibodies (AB3A and Fab fragments) were detected with FITC-conjugated secondary antibodies. For each measurement, 20,000 particles were examined.

from these digested cells using 5 mM phosphate buffer (pH 8) containing E-64 (10 μM) (*9*). The open ghosts were subjected to isotonic conditions (0.14 M KCl, 0.1 M NaCl, 1 mM $MgCl_2$), incubated for 30 min at 37°C, and then washed three times with PBS. The resealed ghosts were suspended in PBS (50% hematocrit). Three volumes of such ghost suspensions were mixed with one volume of antiserum and the mixtures were incubated for 30 min at 37°C. After incubation, the ghosts were washed two times in PBS. These ghosts (10–20 μl at pellets) were gently suspended in PBS (1.4 ml) containing 0.5% (w/v) $C_{12}E_8$, incubated for 10 min at 15°C, and then centrifuged at 20,400 × *g* for 30 min at 4°C. The fluorescence of eosin was measured with excitation at 522 nm and emission at 548 nm using a model FP-750 spectrometer (JASCO, Japan).

Flow Cytometry—For flow cytometric analysis, erythrocyte suspensions exposed to 200 MPa were diluted with PBS. Flow cytometric data were obtained using an EPICS XL-MCL flow cytometer (Coulter, Hileah, FL).

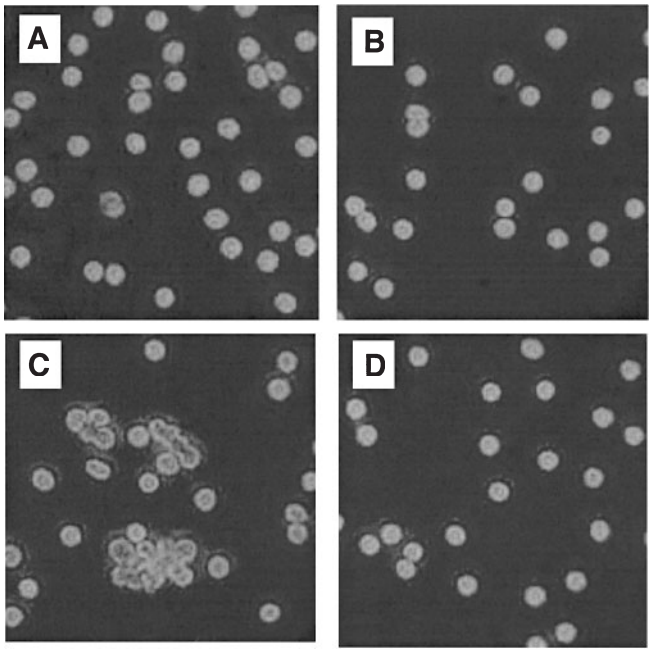


Fig. 2. **Erythrocyte agglutination by antiserum.** A, intact cells in PBS; B, intact cells in antiserum; C, papain-digested cells in antiserum; D, papain-digested cells in antiserum containing Fab fragments. The cells were incubated with antiserum for 1 h at 4°C and observed under a light microscope.

RESULTS

Agglutination of Erythrocytes by Cross-Linking of Band 3 via AB3A—The presence of AB3A molecules in the antiserum was examined by Western blot analysis. Band 3 separated by SDS-PAGE was detected as a single band using the antiserum as a primary antibody and horseradish peroxidase–conjugated goat anti–rabbit IgG as a secondary antibody (Fig. 1A). However, no such band was observed in the absence of the antiserum. This indicates that AB3A molecules are present in the antiserum. The binding of AB3A to the exofacial domain of band 3 in intact erythrocyte membranes or papain-treated ones was examined using a FITC-conjugated secondary antibody against AB3A (Fig. 1B). When the erythrocytes were digested with papain under mild conditions, the proportion of sialic acids removed from the cell membranes was 64.1 ± 3.2% (n = 3). The exofacial domain of band 3 in intact erythrocytes or papain-digested ones was recognized by AB3A molecules. Furthermore, Fab fragments of AB3A also bound to band 3 in papain-digested erythrocytes. However, no FITC fluorescence was observed in the absence of AB3A molecules or their Fab fragments.

The surface of intact erythrocytes is covered with negative charges so that no agglutination of erythrocytes occurs due to electrostatic repulsion (Fig. 2A). When the antiserum containing AB3A was added to intact erythrocyte suspensions, there was no agglutination of cells despite the binding of antibodies to the exofacial domain of band 3, as mentioned above (Fig. 2B). On the other hand, when intact erythrocytes were treated with papain under the same conditions as those in Fig. 1, and incubated with AB3A, cell agglutination was observed (Fig. 2C). However, no such agglutination of papain-digested erythrocytes occurred with Fab fragments of AB3A (Fig. 2D).

Effects of AB3A on High-Pressure–Induced Hemolysis—The effects of AB3A on high-pressure–induced hemolysis were examined using intact erythrocytes. When compressed up to a pressure of 200 MPa, the values of hemolysis for intact erythrocytes were 41.5 ± 2.2% (n = 3), whereas those for antiserum-treated erythrocytes were 35.0 ± 5.2% (n = 3). Thus, the effects of AB3A against high-pressure–induced hemolysis were small in intact

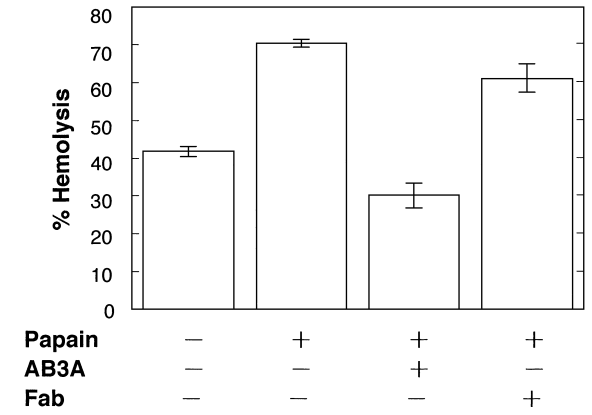


Fig. 3. **Effects of AB3A on hemolysis at 200 MPa of papain-digested erythrocytes.** Erythrocytes were digested with papain (0.5 mg/ml) for 1 h at 4°C. The cells were incubated for 1 h at 4°C with antiserum containing AB3A or Fab fragments. After incubation, the cells were washed and used for pressure experiments. Values are means ± SD for three experiments.

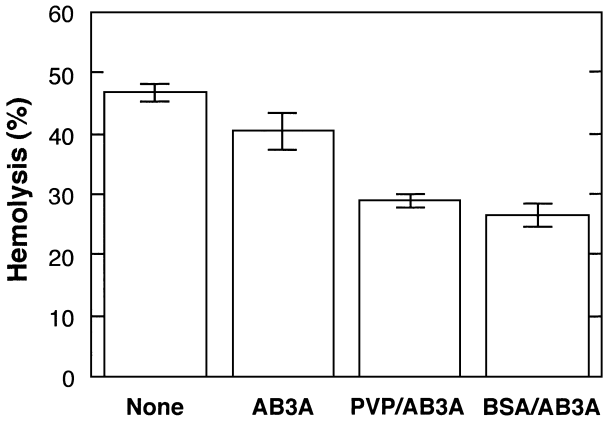


Fig. 4. **Effects of AB3A, PVP, and BSA on hemolysis at 200 MPa of intact erythrocytes.** Intact erythrocytes in PBS were incubated with antiserum in the presence or absence of PVP (50 mg/ml) or BSA (50 mg/ml) for 30 min at 37°C. After incubation, the cells were washed in PBS and subjected to a pressure of 200 MPa. None represents intact cells incubated in PBS. Values are means ± SD for three experiments.

erythrocytes. We previously demonstrated that hemolysis at 200 MPa is enhanced by removing sialic acids from the membrane surface with neuraminidase, trypsin, or chymotrypsin (*8*). Indeed, the values of hemolysis at 200 MPa increased up to 70.2 ± 1.1% (n = 3) upon digestion with papain (Fig. 3). So, the effects of AB3A on such enhancement were examined. The agglutination of papain-treated erythrocytes was observed in the presence of AB3A. When the erythrocyte suspensions containing both agglutinated cells and non-agglutinated ones were exposed to a pressure of 200 MPa, the hemolysis was largely suppressed (Fig. 3). Furthermore, non-agglutinated erythrocytes were separated from agglutinated ones on sedimentation. The values of hemolysis at 200 MPa of non-agglutinated erythrocytes were 52.2 ± 1.0 % (n = 3), suggesting the intracellular cross-linking of band 3 by AB3A. To confirm that such suppression is associated with cross-linking of band 3 by AB3A, papain-digested erythrocytes were exposed to Fab fragments of AB3A. In this case, high-pressure–induced hemolysis was not so suppressed by Fab fragments (Fig. 3). The agglutination of erythrocytes is also induced by zinc ions (Zn^{2+}) (*21*). In such erythrocytes, clusters (or aggregates) of band 3 by $Zn^{2+}$are formed. So, the effects of band 3 clusters on high-pressure–induced hemolysis were examined by the addition of Zn^{2+} to papain-treated erythrocyte suspensions. The values of hemolysis at 200 MPa decreased from 58.1 ± 4.2 (n = 3) to 4.1 ± 4.2% (n = 3) with Zn^{2+} , but returned to 54.0 ± 4.8% (n = 3) upon removal of Zn^{2+} using PB. Here, the reduced effects of papain on hemolysis at 200 MPa may be ascribed to the different buffers (HEPES *vs.* PBS). These results suggest that the enhancement of high-pressure–induced hemolysis on removal of negative charges from the membrane surface is significantly suppressed by extracellular and intracellular cross-linking of band 3 *via* AB3A or by clustering of band 3 *via* Zn^{2+}.

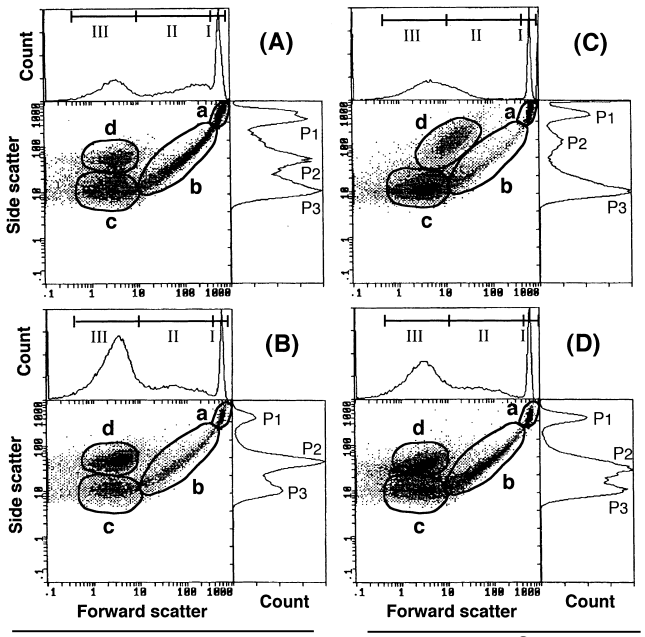
Table 1. **Size and spectrin contents of high-pressure-induced vesicles.**

	Vesicles	
	Diameter (nm)	Spectrin/Band 3
Vesicles (A) from intact cells	423 ± 11	0.63 ± 0.06
Vesicles from papain-digested cells	525 ± 18	0.58 ± 0.02
Vesicles (B) from papain/AB3A-treated cells	520 ± 6	0.59 ± 0.04
Papain digestion of vesicles (A)	410 ± 18	
Papain digestion of vesicles (B)	444 ± 10	

Samples were prepared as described in "MATERIALS AND METHODS." The values of spectrin/band 3 in vesicles released at 200 MPa are given relative to those in ghosts (control). Values are means ± SD for at least two experiments.

We have used papain to remove negative charges on the membrane surface. However, the zeta potential on the membrane surface of intact erythrocytes can also be reduced in the presence of BSA or PVP (*22*). Therefore, it is expected that cross-linking of band 3 by AB3A is readily induced in the presence of BSA or PVP. In fact, the agglutination of intact erythrocytes was observed. This agglutination remained stable during washing with buffer. High-pressure-induced hemolysis of such erythrocytes was also suppressed (Fig. 4). Similar procedures were performed in the absence of AB3A. The values of hemolysis at 200 MPa of erythrocytes treated with BSA and PVP were 54.9 ± 1.3 (n = 2) and 53.1 ± 0.1 % (n = 2), respectively. These values were slightly larger than those (46.1 ± 1.3 %, n = 2) for untreated erythrocytes.

Effects of AB3A on High-Pressure–Induced Vesiculation— When erythrocytes are exposed to a pressure of 200 MPa, membrane vesicles are formed on the cell surface and are partially released from the cell membrane (*9*, *14*). The size of the released vesicles was determined by the light scattering method (Table 1). The size of membrane vesicles released by high pressure increased in papain-digested erythrocytes. A similar size of membrane vesicles was also observed in papain-treated erythrocytes that were subsequently treated with AB3A. In this case, however, the size of vesicles decreased to the control level (444 nm) upon exposure of the vesicles to papain. This suggests that the band 3–containing vesicles released are agglutinated *via* AB3A.



	% Count			
	a	b	c	d
(A)	24.4	34.2	20.7	10.4
(B)	9.7	21.2	17.7	33.9

	% Count			
	a	b	c	d
(C)	18.4	8.2	42.4	14.1
(D)	13.3	24.5	21.7	24.7

Fig. 5. **Dot plots of forward scatter versus side scatter.** Manipulated erythrocytes were exposed to 200 MPa for 30 min at 37°C. After decompression, the cell suspensions were used for flow cytometric analysis. A, intact erythrocytes; B, papain-digested cells; C, cells treated with papain and then antiserum containing AB3A; D, cells treated with papain and then antiserum containing Fab fragments of AB3A. For each measurement, 50,000 particles were examined.

Analysis of the contents or compositions of membrane proteins in vesicles provides the information on membrane protein–protein interactions in erythrocytes (*9*). So, the membrane protein contents in vesicles were analyzed by SDS-PAGE. Under our conditions, band 3 was stable against papain, *i.e.*, no cleaved fragments of band 3 were observed (data not shown). The content of spectrin relative to band 3 in vesicles released from papain-digested erythrocytes or papain/AB3A-treated ones was almost the same as that from intact erythrocytes (Table 1).

Effects of Papain and AB3A on Band 3–Cytoskeleton Interactions—The interactions of band 3 with cytoskeletons were examined using eosin-5-maleimide and a nonionic detergent, $C_{12}E_8$. Eosin molecules bind to lysine-430 of band 3 (*23*). If erythrocyte membranes, in which more band 3 molecules interact with the cytoskeleton, are solubilized by $C_{12}E_8$ and spun down, the pellets may contain more band 3 with cytoskeletal proteins. In this work, the proportions of band 3 interacting with spectrin in intact erythrocytes, papain-digested ones, and papain/AB3A-treated ones were 30.2 ± 1.8 (n = 5), 34.8 ± 1.0 (n = 4), and 33.7 ± 0.4 % (n = 3), respectively. Thus, no effect of papain and AB3A on band 3–cytoskeleton interactions was observed.

Flow Cytometric Analysis of High-Pressure–Treated Erythrocytes—In flow cytometry, forward scatter and side scatter provide information on the size and internal structure of particles, respectively. Intact erythrocytes were exposed to a pressure of 200 MPa and analyzed by flow cytometry. Forward scatter and side scatter were plotted against the number of events. The histogram for forward scatter was divided into three regions (I, II, and III), whereas three peaks (P1, P2, and P3) were observed for side scatter (Fig. 5). Forward scatter *versus* side scatter dot plots were divided into four regions (a, b, c, and d), taking both scatter histograms into consideration. As described previously (*24*), regions a, b, c, and d contain mother cells, fragmented particles, vesicles, and open ghosts, respectively. Here, mother cells are larger ones in pressure-treated cell suspensions but smaller than intact cells in size. Upon exposure to 200 MPa of papain-digested erythrocytes, the number of mother cells decreased, whereas the number of open ghosts increased (Fig. 5B). When papain-treated erythrocytes were incubated with antiserum containing AB3A prior to exposure to 200 MPa, the formation of open ghosts was suppressed and vesiculation was facilitated (Fig. 5C). Here, the different location of region d represents the agglutination of open ghosts. However, such effects as seen with AB3A were not observed with Fab fragments (Fig. 5D).

DISCUSSION

When human erythrocytes are exposed to neuraminidase or proteases such as trypsin and chymotrypsin, high-pressure–induced hemolysis is enhanced (*8*). Similar results were obtained for papain-treated erythrocytes. The values of high-pressure–induced hemolysis increase in proportion to the amount of sialic acids removed from the membrane surface (*8*). Most sialic acids are attached to glycophorins (*25*). These data suggest that glycophorins play an important role in hemolysis at 200 MPa. Here, when we consider the effect of AB3A on high-pressure–induced hemolysis, it is useful to examine band 3–glycophorin interactions. The mobility of band 3 in the erythrocyte membrane is restricted by anti–glycophorin A antibodies (*26*). Wr^b antigen is expressed in the presence of both glycophorin A and band 3 on the erythrocyte membrane (*27*). Additionally, glycophorin A facilitates the expression of human band 3 in the plasma membrane in *Xenopus* oocytes (*28*). These data suggest that glycophorin A is associated with band 3 (*29*). Therefore, cross-linking of band 3 by AB3A may be also affected by the negative charge of glycophorin A. In fact, erythrocyte agglutination, which is induced by intercellular cross-linking of band 3 *via* AB3A, is suppressed by electrostatic repulsion owing to the negative charges of sialic acids on glycophorin A. Similarly, there would be no intracellular cross-linking of band 3 by AB3A. In intact erythrocytes, the suppressive effect of AB3A on hemolysis at 200 MPa is small. On the other hand, in papain-digested erythrocytes or BSA-or PVP-treated ones, negative charges on the membrane surface are reduced so that the intercellular and intracellular cross-linking of band 3 would be readily induced by AB3A. In fact, the existence of agglutinated cells indicates the intercellular cross-linking of band 3 by AB3A. In these agglutinated cells, high-pressure–induced hemolysis is largely suppressed. The hemolysis at 200 MPa of non-agglutinated cells among AB3A-treated cells is significantly suppressed, indicating intra-

cellular cross-linking of band 3 by AB3A. At the present time, we cannot say which cross-linking is more effective for the suppression of high-pressure–induced hemolysis. Similar results of cross-linking of band 3 have been reported for red cell agglutination by concanavalin A (*30*). Concanavalin A exhibits high affinity to the carbohydrates of band 3. Agglutination of erythrocytes by concanavalin A is facilitated by protease treatment of the cells. Additionally, the hemolytic properties under high pressure of concanavalin A–treated erythrocytes are similar to those of AB3A-treated cells (Yamaguchi, T., *et al.*, unpublished observations). The present results indicate that the cross-linking of band 3 *via* AB3A in papain-digested erythrocytes is effective for the suppression of high-pressure–induced hemolysis.

The hemolysis occurring under high pressure is associated with vesiculation (*14*). As with hemolysis, vesiculation starts to occur at about 130 MPa and the amount of released vesicles increases with increasing pressure (*14*). Upon exposure of erythrocytes to 49°C, vesicles are released from the membrane surface of cells (*31*). When 49°C-pretreated erythrocytes are subjected to a pressure of 200 MPa, small vesicles of a diameter of 260 nm are released and the hemolysis is largely suppressed (*9*). Upon exposure to 200 MPa of papain-digested erythrocytes, on the other hand, large sized vesicles (diameter, 525 nm) are released and the hemolysis is enhanced. Such effects of papain digestion are abolished by cross-linking of band 3 *via* AB3A, *i.e.*, vesicles of a diameter of 444 nm are released and the hemolysis is largely suppressed. Thus, the present results also support our hypothesis that high-pressure–induced hemolysis of erythrocytes is suppressed upon a reduction in size of the released vesicles.

In the present work, we have demonstrated that the release of large vesicles followed by enhancement of hemolysis at 200 MPa is observed in papain-treated erythrocytes, and that such properties on vesiculation and hemolysis are affected by AB3A. However, the data for protein extraction with $C_{12}E_8$ and membrane protein contents of released vesicles suggest that the band 3–cytoskeleton interactions are not so perturbed on removal of negative charges or cross-linking of band 3 *via* AB3A. Therefore, the association (or clustering) of band 3 dimers, which are not interacting with the cytoskeleton, may play an important role in the vesiculation and hemolysis occurring under pressure of papain-digested erythrocytes. Clustering of band 3 is proposed to occur upon removal of sialic acids from the erythrocyte membrane surface (*32*). Generally, oligomeric proteins are dissociated under high pressure (*33*). Therefore, the band 3 clusters, which are induced on removal of electrostatic repulsion, also seem to be dissociated under high pressure. It is necessary to examine whether or not the release of large vesicles and the enhancement of hemolysis at 200 MPa in papain-digested erythrocytes are related with the dissociation of band 3 clusters. On the other hand, band 3 clusters are also induced by Zn^{2+} (*21*) or antibodies that recognize the third extracellular loop of band 3 (*34*). However, the response to high pressure of such band 3 clusters is different from that of band 3 clusters formed on removal of sialic acids. When papain-treated erythrocytes are agglutinated with Zn^{2+} or AB3A and then subjected to high pressure, the hemolysis is significantly suppressed and agglutinated cells are observed right after decompression. These results suggest that the band 3 clusters formed by Zn^{2+} or AB3A may be more stable against high pressure, in comparison with the band 3 clusters arising on removal of electrostatic repulsion. Thus, the effects of AB3A on the hemolytic properties at 200 MPa of papain-digested erythrocytes seem to be similar to those of Zn^{2+}. When the aggregates or clusters of band 3 are stable against high pressure, the release of smaller vesicles under pressure is facilitated so that the hemolysis is suppressed.

Interestingly, it has been reported that vesiculation occurs in spectrin/ankyrin- and band 3–deficient erythrocytes (*35*). These deficient cells are unstable in the membrane structure and become spherocytic with the release of membrane vesicles. Thus, hereditary spherocytosis is characterized by the presence of spherocytes in peripheral blood smears. In spectrin/ankyrin-deficient erythrocytes, naturally occurring antibodies bind antigens such as band 3 on the membrane surface and facilitate the vesicle release (*35*). The present results also indicate that the cross-linking of band 3 by its antibody facilitates the vesicle release from erythrocytes under mechanical stress.

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