Role of Hemichrome Binding to Erythrocyte Membrane in the Generation of Band-3 Alterations in β-Thalassemia Intermedia Erythrocytes

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Nine splenectomized, hematologically well-compensated β-thalassemia intermedia patients randomly chosen from a pool of 60 similar patients were studied. Membrane proteins solubilized with non-denaturing detergent C_{14}E_{5} were gel filtered on Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions containing higher than 4,000-kDa molecular-weight aggregates were isolated and analyzed. Four patients had remarkably increased amounts of membrane-bound hemichromes and Igs. In those patients, band 3 underwent oxidative modifications such as aggregation and a decrease in sulfhydryl groups. The other five patients had low amounts of membrane-bound hemichromes and less modifications of band 3. The same band-3 modifications could be reproduced by challenging normal membranes with artificially generated hemichromes or with hemolysates prepared from thalassemic erythrocytes of the high-hemichrome group. Addition of reduced glutathione to the challenged membranes did not hinder hemichrome binding, but prevented oxidative modifications of band 3 and Ig binding to high-molecular-weight band-3 aggregates. Hemichrome binding to band 3, hemichrome-mediated oxidation of band-3 cytoplasmic domains, generation of high-molecular-weight band-3 aggregates, and enhanced opsonization by anti-band-3 antibodies is a possible sequence of events leading to phagocytic removal of erythrocytes in thalassemia.

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MATERIALS AND METHODS

Materials. Rabbit-antihuman and mouse-antirabbit IgG antibodies conjugated to alkaline phosphatase, N-ethyl maleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), and phenylhydrazine were from Sigma Chemical Co (St Louis, MO). Desferrioxamine was from Ciba-Geigy (Milano, Italy). Octaethylene glycol mono-n-dodecyl ether (C_{12}E_{8}) was from Nikkei Chemical Co (Tokyo, Japan). Eosin-5’-maleimide was from Molecular Probes (Eugene, OR). One-biotinamido-4’-(maleimidomethyl)cyclohexane-carboxamide)hydroxylamine (BMCC) and avidin-linked alkaline phosphatase were from Pierce (Rockford, IL). Sepharose CL-6B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Sterile plastics were from Costar (Cambridge, MA). All other reagents were purchased from common commercial sources.

Patients. Five male and four female (age, 18 to 33 years) splenectomized patients affected by β-thalassemia intermedia have been studied. Diagnosis was based on the clinical criterion of transfusion dependency and on genetic analysis. None of the patients had received blood transfusions for at least 2 months. The genotype of the patients was defined by using different techniques, including allele-specific oligonucleotide, restriction enzyme digestion, and allele-
specific amplification as indicated. Blood for normal controls was drawn from apparently healthy laboratory personnel. All blood samples were drawn after informed consent according to protocols approved by University of Milano Ethical Committee. Samples of venous blood (8 to 10 mL) were drawn into heparinized and nonheparinized Vacutainers (Becton Dickinson, Milan, Italy), to obtain erythrocytes and serum, respectively. Samples were kept at 0°C to 4°C and processed within 4 hours. Representative hematologic data obtained by standard techniques and analysis of the hemoglobin β-chain gene mutation of the nine patients are summarized in Table 1.

Preparation of hypotonic erythrocyte membranes, inside-out membranes (IOM), and inside-out vesicles (IOV). Standard hypotonic membranes were prepared at 0°C by hemolysis in hemolysis buffer (5 mM sodium phosphate, pH 8.0) and two washes. To minimize handling artifacts, preparation of hypotonic membranes including three 2-minute centrifugations in refrigerated Eppendorf microfuge was performed in less than 10 minutes. To check band-3 oxidation during handling, hypotonic membranes were also prepared with deoxygenated, helium-flushed hemolysis buffer supplemented or not with reduced glutathione (4 mM/L) or with desferrioxamine (0.1 mg/L). Hemolysis buffer was deoxygenated by sonication and helium bubblingflushing during 30 minutes at 0°C. IOM and IOV were prepared according to Kurot et al and Yuan et al, respectively.

Extraction of erythrocyte membranes with nondenaturing detergent C₆E₄. One milliliter of freshly prepared hypotonic membranes, IOM or IOV, was mixed with 2 mL extraction buffer (130 mM NaCl, 10 mM HEPES, 1 mM EDTA, 1% C₆E₄, pH 7.4) at 37°C for 20 minutes under moderate shaking and then pelleted for 1 minute at 13,000 rpm in an Eppendorf microfuge. The clear supernatant was immediately separated from the pellet and used for gel filtration chromatography.

Gelfiltration chromatography of nondenaturing detergent-extracted membrane proteins. A 100×1.7-cm column was filled with Sepharose CL-6B and equilibrated with a solution containing 50 mM/L NaCl, 10 mM/L HEPES, 0.1% C₆E₄, pH 7.4. Three milliliters of nondenaturing detergent-extracted membrane protein was loaded onto the column and separated at a flow rate of 2 mL/min. The effluent was collected in 5-mL fractions.

Heme-containing compounds were quantified in hypotonic membranes by measuring heme absorbance at 415 nm in the fractions collected after gel filtration chromatography (see above), using millimolar absorptivity value of 131.1 for calculations. Heme-containing compounds were identified as hemoglobin or hemichromes according to their spectral characteristics. Because of the great variability in protein content of membrane in thalassemic subjects, membrane volume was selected as a reference and heme-containing compounds were expressed as nmol heme/mL membrane volume. Membrane volume showed low intersubject variability and roughly corresponded to the original erythrocyte volume in both normal and thalassemic erythrocytes. Comparisons of heme-containing compounds in hypotonic membranes, IOM and IOV, were performed with eosin-5-maleimide-labeled erythrocytes. Labeling with eosin-5-maleimide, a band-3-specific fluorescent label, was performed as outlined. In those studies, eosin-5-maleimide-associated fluorescence was selected as a reference for membrane protein material because large volume differences were found to exist between hypotonic membranes. IOM and IOV. Thus, heme-containing compounds were expressed as the ratio between heme absorbance at 415 nm measured in fractions after gel filtration (see above) and eosin-5-maleimide-associated fluorescence measured in total solubilized proteins before gel filtration.

Quantification of band-3 aggregation. After gel filtration chromatography, band-3-associated eosin-5-maleimide fluorescence was measured in the fractions and the fluorescence value measured in the void volume was normalized to the total fluorescence measured in the fractions.

Assay of erythrocyte-bound Iggs. Erythrocyte-bound Iggs were measured after labeling erythrocytes with rabbit-antihuman IgG antibodies conjugated to alkaline phosphatase as described.

Measurement of band-3-associated sulfhydryl groups by BMCC binding. Hypotonic membranes were prepared as described, but hemolysis buffer at pH 7.0 instead of pH 8.0 was used to increase specificity of BMCC for sulfhydryl groups, according to the manufacturer’s specifications. One hundred microliters of freshly prepared hypotonic membranes were transferred to 1 mL ice-cold hemolysis buffer containing 560 μM/L BMCC, pH 7.0, and then incubated for 10 minutes at room temperature. Membranes were washed twice in hemolysis buffer and nonintegral proteins were stripped by 0.1 N NaOH. After a last wash in hemolysis buffer to remove nonintegral proteins, membranes were solubilized and samples containing the same protein amount (20 mg protein) were separated on adjacent lanes by 8% SDS-polyacrylamide gel electrophoresis. After separation, one lane was stained with Coomassie blue, whereas the other lane was blotted on nitrocellulose and stained with avidin-linked alkaline phosphatase. The staining intensity of the band corresponding to band-3 was quantified by analytical scanning (see above). The staining intensity resulting from alkaline phosphatase activity was normalized to the corresponding Coomassie blue staining intensity and compared in samples from normal controls and from the nine β-thalassemia intermedia patients. The ratio of alkaline phosphatase staining to Coomassie blue staining was found to be constant over a 4-fold dilution range of the samples, indicating that the method was quantitative in the range of concentrations used in the experiments. Oxidation of band-3 during membrane preparation was estimated by comparing band-3-associated sulfhydryls in standard hypotonic membranes, and in membranes prepared with deoxygenated, helium-
flushed hemolysis buffer (see above) supplemented or not with reduced glutathione or desferrioxamine.

Preparation of hemichromes and erythrocyte lysates. Hemichromes were produced by treating hemoglobin with phenylhydrazine and isolated. Erythrocyte lysates were prepared by lysing normal or thalassemic erythrocytes from high-hemichrome patients (group B patients, see Fig 2) with 20 vol of hypotonic buffer. Membranes were pelleted by centrifugation, hemoglobin concentration was brought to 20 mg/mL by brief vacuum centrifugation at 4°C, and the supernatant was used immediately.

Challenge of normal hypotonic membranes with hemichromes or erythrocyte lysates. One milliliter freshly prepared normal hypotonic membranes was incubated with 2 mL erythrocyte lysate containing 20 mg/mL hemoglobin or with hemolysis buffer containing 20 mg/mL hemichromes. After 30 minutes at 4°C, 0.3 mL of 10-fold-concentrated phosphate-buffered saline (PBS) was added and the membranes incubated for 30 minutes at 20°C. Membranes were washed three times in cold PBS, and divided in two aliquots. A first aliquot was resuspended in PBS containing 10% fresh autologous serum, incubated 30 minutes at 4°C, washed three times with cold PBS and further incubated in 10 volumes PBS containing rabbit-antihuman IgG antibodies conjugated to alkaline phosphatase (diluted 1:2,000) for 30 minutes at 4°C. After five washes with cold hemolysis buffer, membrane proteins were solubilized in extraction buffer containing the nondenaturing detergent C,2E8 (1.5%) and gel filtered as described above. Membrane-bound Igs and hemichromes were measured in the fractions as described. A second aliquot was used for measurement of band-3-associated sulfhydryl groups as described above. When needed, 4 mmol/L reduced glutathione was added to the hemichrome solution or erythrocyte lysate.

RESULTS

Membrane-bound hemichromes and Igs in β-thalassemia intermedia patients. Nine splenectomized patients randomly selected from a group of 60 β-thalassemia intermedia patients were studied. Hypotonic membranes of four patients retained visibly high amounts of heme, even after several washes in hypotonic buffer. To exclude that heme-containing compounds were retained because of membrane resealing, hypotonic membranes of those four patients were solubilized in non-denaturing detergent C,2E8 and bound heme-containing compounds separated from free globin by gel filtration chromatography. As shown in Fig 1, heme-containing compounds were found in peak A, corresponding to the column void volume (>4,000 kD molecular weight), indicating heme binding to aggregated, high-molecular-weight membrane components. Heme-containing compounds were also present in peak B (<100 kD molecular weight), indicating that large amounts of retained hemoglobins were not tightly bound to the membrane. The absorption spectrum of peak
A (Fig 1, right panel) strongly resembled that of hemichrome. For this reason, the heme-containing compounds found in peak A are referred to as hemichromes. The absorption spectrum of peak B (Fig 1, right panel) was identical to that of oxyhemoglobin.

Heme present in peak A could also be caused by trapping of large aggregates of denatured hemoglobin (Heinz bodies) within resealed erythrocytes. To check this possibility, hypotonic membranes, IOVs, and IOMs prepared from patient 3 of the low-hemichrome group and from patients 6 and 7 of the high-hemichrome group (see below) were compared. Due to the distinctly different volume of IOM and IOV, eosin-5-maleimide fluorescence measured in total solubilized proteins before gel filtration was used as a reference. Eosin-5-maleimide, a specific band-3 fluorescent label, reliably reflects total amount of solubilized membrane protein. Solubilized membranes were gel filtered and fractions were analyzed for heme content. In peak A, corresponding to the column void volume, the ratio between band 3-associated sulfhydryls, membranes were depleted of nonintegral proteins by alkali treatment and sulfhydryl groups were 4.5% (mean ± SD) (patients 1 to 5) and group B (patients 6 to 9) had high amounts of bound hemichromes (4.58 ± 1.36 nmol/mL membranes [mean ± SD]) (Fig 2). The difference between group A and B was highly significant (P < .001).

Because membrane-bound hemichromes and coclustered band 3 are expected to promote binding of Ig, membrane-bound Igs were measured in all patients. The same bimodal distribution was noted for membrane-bound hemichromes as was noted for aggregated band 3. Constantly, group B patients had higher (2.9- ± 1.07-fold [mean ± SD], n = 4) membrane-bound Igs than group A patients. As for hemichromes and aggregated band 3, the difference between group A and B was highly significant (P < .001).

**Band-3-associated sulfhydryl groups and aggregated band 3 in β-thalassemia intermedia patients.** To measure band-3-associated sulfhydryls, membranes were depleted of nonintegral proteins by alkali treatment and sulfhydryl groups labeled with BMCC, a biotinylated maleimide derivative. After labeling, membrane proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted on nitrocellulose, and labeled with avidin-linked alkaline phosphatase. This representative experiment indicates visually that high-hemichrome (group B) erythrocytes had less BMCC-reactive band-3-associated sulfhydryl groups than normal erythrocytes. Quantitative comparison of scans shows that group B membranes had 26.8% ± 7.3% (mean ± SD; n = 4) less band-3-associated sulfhydryl groups than control membranes (P < .01). In group A membranes, band-3-associated sulfhydryl groups were 4.5% ± 3.9% (mean ± SD; n = 4) less (difference not significant) than in control membranes.

Labeling of band 3 with eosin-5-maleimide allowed quantitation of aggregated band 3 present in the column void volume. The percentage of aggregated band 3 was 0.3% ± 0.24% (mean ± SD; n = 5) of total band 3 in control erythrocytes, 4.9% ± 3.14% (mean ± SD; n = 5) in group A patients, and 26.4% ± 11.5% (mean ± SD; n = 4) in group B patients. The difference between group A and group B was highly significant (P < .001).

To check occurrence of artefactual oxidation of band-3-associated sulfhydryl groups during handling, erythrocytes were processed within 4 hours from withdrawal and the time for hypotonic membrane preparation until membrane labeling with BMCC was not longer than 10 minutes. The amount of band 3 oxidation during membrane preparation was estimated by comparing band-3-associated sulfhydryls in standard hypotonic membranes and in membranes prepared with deoxygenated, helium-flushed hemolysis buffer supplemented or not with reduced glutathione and desferrioxamine. In membranes of one group B patient, the percent decrease in band-3-associated sulfhydryl groups relative to control membranes was 24% in standard mem-

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**Fig 3.** Visualization of band-3-associated sulfhydryl groups in membrane isolated from BMCC-labeled erythrocytes and separated by SDS-polyacrylamide gel electrophoresis. Lanes 2 and 4 are membrane proteins isolated from high-hemichrome (group B) β-thalassemia intermedia erythrocytes. Lanes 1 and 3 are membranes isolated from a normal control. Lanes 1 and 2 were stained with Coomassie blue, lanes 3 and 4 were stained with avidin-linked alkaline phosphatase. This representative experiment indicates visually that high-hemichrome (group B) erythrocytes had less BMCC-reactive band-3-associated sulfhydryl groups than normal erythrocytes. Quantitative comparison of scans shows that group B membranes had 26.8% ± 7.3% (mean ± SD; n = 4) less band-3-associated sulfhydryl groups than normal membranes (P < .01). In group A, band-3-associated sulfhydryl groups were 4.5% ± 3.9% (mean ± SD; n = 4) less than in normal membranes (difference not significant).
branes, 29% in reduced glutathione-supplemented membranes, 20% in desferrioxamine-supplemented membranes. The comparison was repeated in two additional patients with similar results (not shown). These data indicate that no oxidation of band-3–associated sulfhydryls occurred during sample handling in thalassemic membranes.

Effects of hemichrome challenge in normal hypotonic membranes. To show that membrane-bound hemichrome may be responsible for oxidative damage to the membrane and for enhanced Ig binding, normal hypotonic membranes were challenged with artificially generated hemichromes or with membrane-free lysates obtained from group B erythrocytes and were successively opsonized with autologous serum. As shown in Table 2, challenged membranes contained less band-3–associated sulfhydryls and increased amounts of membrane-associated hemichromes and IgGs. Reduced glutathione added during the membrane challenge did not influence the formation of the hemichrome-containing aggregate, but decreased IgGs associated to the aggregate by fivefold and almost normalized band-3–associated sulfhydryls. As expected, incubation of normal membranes with lysates prepared from normal erythrocytes or group A thalassemic erythrocytes (not shown) had no effect on the parameters considered.

DISCUSSION

Modifications of band 3 are central in the generation of phagocytic recognition and removal signals in naturally senescent20–23 and variously altered erythrocytes.24–26 Clustering of band 3 is considered to be an early event after oxidative insult leading to deposition of autologous antibodies with anti–band-3 specificity, limited activation of the complement cascade, increased binding of complement C3 fragments, and final enhancement of phagocytosis.22,23,26,29

Recently, hemichrome binding to the erythrocyte membrane, band-3 aggregation and deposition of Ig and complement fragments were observed in two patients with β-thalassemia intermedia.9 However, no indications were given on the significance of the observed band-3 changes in thalassemia intermedia at large, on the mechanism of band-3 alterations and on the role of hemichrome binding.

To address those questions, we have studied nine splenectomized patients of both sexes affected by β-thalassemia intermedia, randomly chosen from a pool of 60 similar patients. All patients were hematologically well compensated. Clinical and hematologic data and the nature of the mutation indicate marked heterogeneity in the patients and no evident correlation with any of the biochemical parameters studied. However, taking the amount of membrane-bound hemichromes as the discriminant, two clearly distinguished groups seemed to exist. Group A (five of nine patients) had low amounts of hemichromes, whereas group B (four of nine patients) had high levels of hemichromes bound to the erythrocyte membrane. This bimodal distribution allowed establishment of correlations to other biochemical indices of erythrocyte damage, such as membrane protein aggregates, band 3 oxidation, and deposition of Ig on the erythrocyte membrane.

Deposition of Ig was increased and band 3 underwent oxidative changes only in erythrocytes with high amounts of membrane-bound hemichromes (group B). Band 3 changes included high–molecular-weight band-3 aggregates in the membrane and decreased band-3–associated sulfhydryl groups. Group A erythrocytes had low amounts of membrane-bound hemichromes and IgGs, and distinctly less evident biochemical alterations of band 3.

Experiments performed with group A and group B erythrocytes indicate that approximately the same amount of hemichromes was stably associated to membrane proteins prepared either from hypotonic membranes or IOMs. This excludes the artefactual interference of large Heinz body–like aggregates trapped within resealed membranes. Artefactual origin of oxidative damage to band 3 during sample handling was also excluded by experiments performed with hypotonic membranes prepared with deoxygenated buffer supplemented with desferrioxamine or reduced glutathione.

There was an evident correlation between the amount of membrane-bound hemichromes and band-3 alterations. A selective high-affinity binding site for hemichromes is present on the cytoplasmic domain of band 3, and membrane-bound hemichromes (possibly associated with small amounts of free iron25) generate reactive oxygen species (ROS).1
data basically agree with the chain of events known to occur to band 3 after application of oxidant agents to normal erythrocytes: disulfide cross-linking of cytoplasmic domain cysteins, reorientation of intramembrane and extracellular domains, band-3 aggregation, and exposure of putative anti-band-3 epitopes at proper distance and orientation as to allow bivalent antibody binding.12

The above sequence and the causal role played by hemichromes are confirmed by the observation of the basic pattern of band-3 alterations in normal erythrocyte membranes challenged with artificially generated hemichromes or hemolysates isolated from group B erythrocytes and by the fact that addition of reduced glutathione did not hinder hemichrome binding to the membrane, but prevented both oxidation of band-3-associated sulfhydryls and Ig binding to band-3 aggregates.

In comparing mild oxidation brought about by diamide with the thalassemia data, one should note important differences. First, diamide aggregated only about 1% of total band 3,12 whereas in thalassemia 26% of band 3 was clustered in high–molecular-weight aggregates; second, diamide elicited binding of relatively small amounts of autologous Ig/complement,12 whereas binding of both opsonins was quite massive in thalassemia; third, stimulation of phagocytosis after diamide12 was less pronounced than in thalassemia (Manne F, Cappadoro M, Giribaldi, Arese P, Turrini F: manuscript in preparation). Evidently, the combined oxidant action of hemichromes and the attachment of denatured globin components that constitute Heinz bodies potentiate each other and powerfully enhance opsonization in thalassemic erythrocytes.

Interestingly, in addition to thalassemia,1,8 and erythrocyte senescence,23,25,26 several other hemolytic conditions (falciparum in G6PD deficiency,27,31,32 malarial anemia,29 sickle cell anemia,13,24 congenital Heinz body hemolytic anemia33) appear to be characterized by oxidative membrane modifications and hemoglobin denaturation. Therefore, the present study may deal with a general mechanism that could play a role in physiologic or pathologic erythrocyte removal.

Our data do not elucidate two important issues noted here. First, it is not clear why about half of the patients had high levels of membrane-bound hemichromes and correspondingly severe band-3 alterations, whereas the other half of the patients had almost unaltered erythrocytes; second, it is not clear why patients with less severely altered erythrocytes (group A) were more anemic, had lower erythroblast numbers and smaller mean corpuscular volume than group B patients.

As to the first point, the amount of free unpaired α chains, free heme, or free hemichromes was possibly higher in the cytoplasm of group B patients. Indeed, challenge of normal membranes with membrane-free lysates from group B erythrocytes elicited the same band-3 alterations observed after hemichrome challenge. Challenge of normal membranes with membrane-free lysates from group A erythrocytes had no effect.

As to the second point, it should be remembered that 80% to 90% of senescent or altered erythrocytes is removed extravascularly by macrophages.34 Thus, the efficiency of the phagocytic system is important to determine how many erythrocytes and which kind of erythrocyte is removed. In other words, a low-efficiency or more tolerant phagocytic system would leave even opsonized and damaged erythrocytes undisturbed as it occurs in group B patients, who were indeed less anemic than group A patients. Functionality of the phagocytic system in thalassemia patients certainly deserves consideration in future studies.

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