Erythrocyte Membrane Skeleton Abnormalities in Severe $\beta$-Thalassemia

By E. Shinar, O. Shalev, E.A. Rachmilewitz, and S.L. Schrier

The protein composition of ghosts, inside-out vesicles (IOV), and membrane skeletons (MS) of erythrocytes (RBC) from splenectomized (spx) and nonsplenectomized (non-spx) patients with $\beta$-thalassemia major and $\beta$-thalassemia intermedia was determined. Ghosts from spx thalassemia intermedia patients had a significant increase in their globin content (which was mostly heme reactive) and contained extra polypeptides in the protein 4.2 to 5 and 6-globin areas. The Triton-extracted MS from all of the thalassemic patients showed two major abnormalities: (a) they retained up to twice the amount of protein 3 when compared with controls; (b) they had a significant increase in their globin content, the concentration of which was independent of their protein 3 content. Analysis of the IOV revealed no differences between those prepared from normal controls and those of the patients. MS from spx thalassemia intermedia patients were grossly abnormal when examined by scanning electron microscopy and they exhibited aggregates of material that on transmission electron microscopy suggested the presence of globin precipitates. We propose that, although the integral protein composition, as reflected in the IOV, from severely affected $\beta$-thalassemics is intact, their MS assembly is deranged. The altered skeletal structure of thalassemic RBC could result from attachment of denatured globin to the skeleton components. These abnormalities may contribute to the premature cell death seen in severe $\beta$-thalassemia.

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Materials and Methods

Patients Studied

Samples of heparinized venous blood were collected from a total of 18 patients with $\beta$-thalassemia intermedia, five patients with $\beta$-thalassemia major, five patients with other hemoglobinopathies, and four nonhemoglobinopathic reticuloocyte-rich patients as well as 14 normal splenectomized and nonsplenectomized subjects under protocols approved by the Hadassah Hospital Committee on Human Experimentation and the Stanford Committee on Human Experimentation. The majority of the thalassemic patients were of either Kurdish-Jewish or Israeli-Arabic extraction. Eleven of the 18 patients had been splenectomized (spx) several years prior to the present study whereas the rest were nonsplenectomized (non-spx).

Initial samples were studied in Jerusalem within 24 hours after blood collection. In order to confirm reproducibility, to apply more stringent inhibition of proteolysis, and to utilize newer methods of quantifying polypeptide composition, most patients were restudied at Stanford. Heparinized whole blood samples arrived at Stanford within 36 hours after being drawn. Upon arrival they were washed, and the buffy coat was removed. The hematocrit value was brought to 30% with isotonic saline, to which 2 mmol/L diisopropyl fluorophosphate (DFP) and 10 $\mu$g/mL of pepstatin A and of leupeptin were added to prevent proteolysis. The thalassemia intermedia patients rarely required blood transfusions, and none had been transfused 2 to 3 months prior to study. The thalassemia major patients were obtained just prior to the next transfusion, typically 4 weeks after the last transfusion. Each sample was not subjected to all of the analyses eventually used. The detailed hemoglobin and globin chain synthesis ratios of most of the patients have been previously reported.

Preparation of Ghosts and Membrane Fractions

White ghosts were prepared by lysis of RBC in hypotonic phosphate buffer according to the method of Dodge et al. The lysis...
buffer contained 1 mmol/L EDTA and 0.1 mmol/L phenylmethylsulfonyl fluoride to inhibit protease activity (0.2 mmol/L DFP was used for the shipped samples). Ghosts prepared from the spx thalassemia intermedia patients retained a brownish color despite extra washes with 40-fold volumes of lysis buffer. IOV were prepared by incubating white ghosts in 0.5 mmol/L sodium phosphate buffer (pH 8) at 0°C for one hour and then harvesting them at the interface of a Dextran T-500 barrier (Pharmacia Fine Chemicals, Piscataway, NJ) by centrifugation in a Spincro SW41 rotor (Spinco-Beckman, Palo Alto, CA) for two hours at 40,000 rpm.12 MS were prepared by direct lysis of intact RBC (pretreated both in Jerusalem and Stanford with 2 mmol/L DFP, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin) with Triton X-100 at a concentration of 25 mg/mL packed RBC as described by Sheetz.13 Since considerable care is necessary in standardizing this preparation, the Triton was weighed out, and duplicate microhematocrits were performed on the packed RBC to insure that the ratio of Triton to RBC volume was tightly controlled. The MS were extracted by using both isotonic and hypertonic 0.3 mol KCl buffer. To control proteolysis further the extraction buffers and sucrose gradients contained 0.25 mmol/L DFP, 10 μg/mL pepstatin A, and 10 μg/mL of leupeptin. The addition of hypertonic KCl removed considerably more integral proteins and produced MS containing substantially less protein.13 The amount of protein 3 retained in these MS was calculated as a percentage of the total protein after subtracting the globin and protein 6-globin content in each sample to provide a more equivalent denominator for comparative purposes. On several occasions, cellulose fiber columns were used to remove the WBC and platelets.14 No differences in the results were found when this time-consuming step was used, and therefore it was not used routinely.

When it became apparent that MS from spx β-thalassemia intermedia RBC contained more globin than the non-spx MS, it was important to determine whether that globin was firmly adherent to the MS or simply reflected aggregates of globin enmeshed or trapped in the cytoskeleton at the time of Triton skeleton formation. To explore this possibility, MS from spx β-thalassemias were frozen once at −20°C and then thawed by warming up to room temperature slowly. Aliquots were set aside, and the remainder was sonicated for three to five minutes at output control 3 in the probe sonicator Ultrasonics, Inc, Model W-220 (Orangeburg, NY). All samples were then recentrifuged at 100,000 g for 60 minutes, and the pellets were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as will be described later.

**Protein Determination**

Protein was determined by the Bradford method,15 with bovine serum albumin as a standard, and by the method of Lowry et al.16 In an attempt to distinguish free globin from heme-containing globin, the hemoglobin content of the several membrane preparations was determined in Jerusalem by using the benzidine reaction.17

**SDS-PAGE**

Samples were separated on 6% to 18% nonlinear gradient slab gels according to the method of Laemmli.18 The gels were stained with Coomassie blue or PAS reagent. In Jerusalem the gels were scanned and analyzed in an automated standardized Helena Quick Quant II densitometer (Helena Laboratories, Beaumont, TX). The area occupied by each band was determined with a recording integrator. The protein composition of the gels of the samples studied at Stanford was determined by the method described by Agre et al.19 Briefly, Coomassie blue-stained bands were cut out, eluted with 1 mL of 25% pyridine, and the absorbance at 605 nm was measured. This method yielded reproducible quantitation of the spectrin:protein 3 ratios and supported the densitometric determinations.

**Immunoreactive Spectrin**

To determine whether some of the polypeptide bands that appeared in excess in the thalassemic ghosts and MS were products of proteolytic attack on spectrin we used the technique of Western immunoblotting. Proteins from SDS-PAGE were transferred to nitrocellulose paper exactly as described by Towbin et al.20 Then after unoccupied sites were covered with albumin, the nitrocellulose was reacted with our polyclonal-monospecific rabbit antispectrin antibody,21 and the sites of binding were identified by the immunoperoxidase reaction.22 Screening studies had established that with the use of this technique our monoclonal mouse antispectrin antibody reacted only with β-spectrin,23 whereas the rabbit polyclonal antibody reacted only with α- and β-spectrin at a ratio of roughly 80:20. These studies were done only on the samples sent to Stanford.

**Electronmicroscopic Studies**

Both white ghosts and MS were examined by transmission electron microscopy (TEM) in a Philips EM-300 electron microscope (Philips, Mahwah, NJ) after fixing with a 2% solution of glutaraldehyde in phosphate-buffered saline and then embedding and sectioning.24 Studies by the scanning electron microscope (SEM) were performed after processing of the samples according to the method of Sanders et al25 using a Jeol-35 scanning electron microscope (Jeol USA, Inc, Peabody, MA).

**Statistical Analysis**

Statistical analysis was carried out using the Abstat program (Abstat-Anderson-Bell Co, Canon City, CO) for the IBM-PC (IBM, Boca Raton, FL).

**RESULTS**

**Polypeptide Analysis**

**Hypotonic ghosts.** In the spx thalassemia intermedia ghosts studied at Stanford there was an increase in the globin content that reached 13.7% ± 3.5% (mean ± S.D.) of the total amount of protein compared with 3.0% ± 1.6% and 4.7% ± 2.7% in the non-spx thalassemics and normal controls, respectively (P < 0.001) (Table 1, Fig 1). Studies performed in Jerusalem yielded similar results (8.9% ± 0.7%, 3.7% ± 0.2%, and 2.9% ± 1.0%, respectively). The benzidine reaction performed on the samples in Jerusalem showed that the majority of this membrane-associated globin was heme containing (8.2% ± 4.4%, 3.8% ± 2.8%, and 2.9% ± 1.2%, respectively). In addition, extra polypeptides appeared in the ghosts from spx thalassemia intermedia patients in the areas between proteins 4.2 to 5 and 6-globin. The increase in the 4.2 to 5 area was significantly greater in spx patients than in the comparable controls (P < 0.03), but there was no significant difference between controls and non-spx thalassemics (P = .3). The increase in the 6-globin area in spx patients was significantly greater than either controls (P = .0003) or non-spx thalassemics (P = .0006); however, there was no significant difference between controls and non-spx thalassemics (P = .26).

Although the changes in polypeptide pattern in the 4.2 to 5 area and the 6-globin area seem to be present in other hemoglobinopathies as well, the increase in globin content was highest in the spx β-thalassemics. The spectrin:protein 3 ratio was slightly lower in the spx thalassemics than in either
Table 1. Analysis of SDS-PAGE of Ghosts of Thalassemia Intermedia Patients and Controls

<table>
<thead>
<tr>
<th></th>
<th>Spectrin/Protein 3 Ratio</th>
<th>Globin*</th>
<th>4.2-5*</th>
<th>6-Globin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls (6)</td>
<td>1.35 ± .10</td>
<td>4.7 ± 2.7</td>
<td>9.5 ± 2.1</td>
<td>5.7 ± 1.3</td>
</tr>
<tr>
<td>Thalassemia intermedia, non-spx (7)</td>
<td>1.32 ± .20</td>
<td>3.0 ± 1.6</td>
<td>10.9 ± 2.9</td>
<td>6.7 ± 1.7</td>
</tr>
<tr>
<td>Thalassemia, spx (11)</td>
<td>1.15 ± .16</td>
<td>13.7 ± 3.5</td>
<td>11.9 ± 2.0</td>
<td>13.0 ± 3.6</td>
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Other hemoglobinopathies

<table>
<thead>
<tr>
<th></th>
<th>Glowbin*</th>
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<tr>
<td>Sickle cell anemia</td>
<td>A: 7.4</td>
</tr>
<tr>
<td></td>
<td>B: 6.1</td>
</tr>
<tr>
<td>C: 6.1</td>
<td>C: 15.2</td>
</tr>
<tr>
<td>Hgb SC: 6.2</td>
<td>11.3</td>
</tr>
<tr>
<td>Hgb AC: 3.9</td>
<td>9.9</td>
</tr>
</tbody>
</table>

NOTE. Numbers in parentheses are the number studied. Expressed as a percentage (mean ± SD) of the total protein.

controls (P = .01) or non-spx thalassemics (P = .05), and there was no difference between controls and non-spx thalassemics (P = .7). Immunoblot analysis indicated that in the ghosts of each of the spx thalassemic patients there was a very faint band in the protein 7 area that reacted with antispectrin antibodies (data not shown). No spectrin immunoreactivity was detected in the globin, the 4.2 to 5, and the 6-globin areas.

IOV. No difference was found in the SDS-PAGE analysis of IOV obtained from thalassemia intermedia patients (spx and non-spx) and normal controls. Notably, no excess accumulation of globin was found in thalassemic IOVs (data not shown).

MS. When extracted under isotonic conditions the globin content of MS from the spx thalassemic patients was significantly higher (11.6% ± 7.0%) than that from the non-spx thalassemia intermedia patients (4.3% ± 0.7%) and the normal controls (2.8% ± 0.5%) (P < .001) (Table 2, Fig 2). The benzidine reaction revealed that approximately 75% (8.8% ± 1.8%) of the globin attached to the spx thalassemic MS contained heme.

The large amount of globin in spx thalassemic MS was unanticipated, and it was therefore important to determine whether the globin was associated with the cytosol face of protein 3.24 The isotonic Triton extraction leaves considerable protein 3 attached to the membrane skeleton.25 Therefore, RBC MS were prepared in Triton in the presence of hypertonic KCl buffer, thus producing MS containing substantially less protein 3.22 The increased removal of protein 3, however, was not followed by any decrease in the globin content of the thalassemia intermedia MS, which was again elevated, particularly in the spx thalassemics’ MS, rising to 14.0% ± 5% and to 7.2% ± 2.1% in the non-spx thalassemic MS.

The globin could be firmly bound to the MS, or aggregates of cytosolic or membrane globin could have been nonspecifically enmeshed or trapped during the formation of Triton membrane skeletons. To test these possibilities MS from spx thalassemics were frozen at −20°C, thawed once, and then divided into two aliquots, one of which was saved for analysis and the other subjected to 5 seconds of sonication (see Materials and Methods). Both samples were then centrifuged and the pellets analyzed by SDS-PAGE. In one of three such experiments the globin content was initially 12.2% of the membrane protein. After freezing and thawing once, the globin content expressed as a percentage of the total protein (mean ± SD).

<table>
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<tbody>
<tr>
<td>Normal Controls</td>
<td>2.8 ± 0.5*</td>
<td>2.5 ± 1.4</td>
</tr>
<tr>
<td>Thalassemia intermedia, non-spx</td>
<td>4.3 ± 0.7</td>
<td>7.2 ± 2.1</td>
</tr>
<tr>
<td>Thalassemia intermedia, spx</td>
<td>11.6 ± 7.0</td>
<td>14.0 ± 5.0</td>
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Other hemoglobinopathies

<table>
<thead>
<tr>
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<tr>
<td>Sickle cell anemia</td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10.6</td>
<td></td>
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<tr>
<td>C</td>
<td>4.6</td>
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<tr>
<td>Hgb SC</td>
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<td>Hgb AC</td>
<td>7.1</td>
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*Globin content expressed as a percentage of the total protein (mean ± SD).
Fig 2. SDS-PAGE gels of RBC membrane skeletons extracted under hypertonic conditions. Lanes A and E are controls, B and D are spx thalassemias, and C is a non-spx thalassemic. Lane G contains normal ghosts for reference. The numbering system on the right-hand margin is the same as in Fig 1.

The globin represented 14.1% of the membrane protein, and after sonication the globin content was 13.2% of the membrane protein. The proportion of the other membrane proteins (ie, proteins 1, 2, 3, 4, 1, and 5) was unchanged by either freezing and thawing or freezing and sonication (data not shown). In performing these experiments it became apparent than under these controlled conditions the protein 3 content of thalassemic MS was increased (Fig 2). Studies were therefore extended to include five transfusion-dependent thalassemia major patients, the blood samples being obtained just prior to transfusion. The reticulocyte-rich controls were patients with hemolysis due to vasculitis (reticulocytes, 6.9%), non-spx thalassemics, and C is a non-spx thalassemic. The morphological changes consisted of a thick and coarse mesh that contained large excrescences. When analyzed by TEM (Fig 4), these bumps were seen to consist of electron-dense material that was unevenly distributed in the spx thalassemic MS and had an appearance similar to that described for Heinz bodies.

**Table 3. Protein 3 Content of MS Prepared in the Presence of Hypertonic KCl**

<table>
<thead>
<tr>
<th>Protein 3 (%)</th>
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<tbody>
<tr>
<td>Normal subjects (B)</td>
<td>12.6 ± 1.8</td>
</tr>
<tr>
<td>Reticulocyte-rich patients (5)</td>
<td>9.9 ± 2.2</td>
</tr>
<tr>
<td>𝛽-Thalassemia intermedia (4 spx, 3 nonspx)</td>
<td>16.6 ± 1.8</td>
</tr>
<tr>
<td>𝛽-Thalassemia major (5)</td>
<td>17.9 ± 2.7</td>
</tr>
<tr>
<td>SS-1</td>
<td>8.6</td>
</tr>
<tr>
<td>SS-2</td>
<td>9.9</td>
</tr>
<tr>
<td>SS-3</td>
<td>7.2</td>
</tr>
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</table>

NOTE. Values are calculated as a percentage of the total membrane protein less the globin and protein 6-globin content (mean ± SD). The numbers in parentheses are the number of subjects studied.

**DISCUSSION**

The 𝛽-thalassemic RBC provides an example wherein events inside red cells lead to the premature removal of affected RBC. We postulate that the unmatched 𝛼-globin aggregates lying at the membrane cytosolic face cause an alteration transmitted to the external membrane surface that eventuates in macrophagic attack and removal of the affected cells. The signal to the macrophage might be complex. There is known to be a decrease in thalassemic membrane sialic acid residues that is unevenly distributed. It has been recently shown that after the removal of the sialic residues there is exposure of the underlying galactosyl residues to which the naturally occurring antigalactosyl antibodies bind, thus providing an Fc signal to macrophages. Reduced RBC deformability has also been described in thalassemia, and such a change could, by prolonging the contact between damaged RBC and the macrophage, result in greater opportunity for macrophagic attack. RBC rigidity in thalassemia is not caused by an increased mean corpuscular hemoglobin concentration or by a reduced surface area-to-volume ratio but is most likely caused by an altered membrane modulus. This change in membrane function could also be caused by the accumulation of 𝛼-globin aggregates at the membrane cytosol face that affect either integral membrane or MS proteins or both.

In this study as in prior studies, SDS-PAGE analysis of RBC ghosts from spx thalassemic patients revealed an increase in their globin content as compared with the non-spx patients and normal controls (Table 1, Fig 1). In addition, extra polypeptides in the protein 4.2 to 5 and 6-globin areas appeared in the ghosts from the spx thalassemia intermediate RBC and were free of spectrin degradation products as...
detectable by the Western immunoblot technique. The appearance of these extra polypeptides occurred despite the regular use of comprehensive antiproteolysis measures. These polypeptides could result from attachment of cytoplasmic proteins such as catalase to the thalassemic membranes.

Alternatively some of these bands could represent dimers, trimers, or even tetramers of native or degraded hemoglobin. Allen and Cadman have shown in an in vitro model that an increase in calcium uptake by RBC induces similar changes in their membrane polypeptides. We have recently demonstrated that there is an increased calcium content in β-thalassemia intermedia RBC that was most pronounced in the spx patients. It is therefore possible that calcium plays an important role in thalassemic RBC that is apparent despite the rigorous antiproteolysis measures taken. We compared the changes in spx thalassemic ghosts with the ghosts from patients with other hemoglobinopathies. The increase in polypeptides in the 4.2 to 5 and 6-globin areas seems to be nonspecific. However, the elevation in globin content in the spx thalassemic is considerably greater than the values seen in three patients with sickle cell anemia who are thought to be hyposplenic by conventional clinical criteria.

Resolution of membranes into their integral proteins (ie, IOV) and MS components revealed that there were no differences in IOV from thalassemia intermedia patients (spx or non-spx) and controls. The absence of globin from IOVs prepared from spx thalassemia intermedia patients suggested either that the globin was not attached to integral proteins or lipids or that it could be removed by low-ionic strength media. Surprisingly it was the MS from the spx thalassemia intermedia patients that contained significantly increased amounts of globin at a level that was approached by only one of the three sickle cell anemia patients studied (Table 2). This globin could not be removed by fairly drastic physical means consisting of freezing and thawing with or without subsequent sonication. The proportion of other membrane proteins, particularly protein 3, was also not altered by these treatments. Therefore it is likely that the globin and other perhaps associated membrane constituents are firmly bound to membrane skeletons. Protein 3 is known to be one of the membrane binding sites for hemoglobin and for hemichromes. Therefore we used hypertonic KCl to increase the Triton extraction of protein 3 from thalassemic MS. However, this maneuver was not followed by any decrease in globin content (Table 2). Thus it is possible that cytoskeletal proteins serve as an attachment site for globin aggregates. Snyder et al reported that during in vivo senescence there is increased binding of globin chains to spectrin, and it has been hypothesized that it is the α-chain of hemoglobin that binds to the α-chain of spectrin. However, our data do not identify the site(s) of attachment of the putative α-globin aggregates to the membrane skeleton. The idea that association of globin aggregates to MS components occurs in spx thalassemies is further supported by the distinctly abnormal morphology of the MS from spx thalassemics. On SEM the MS from spx thalassemia intermedia patients displayed coarse stranding and bumps (Fig 3), and on conventional TEM, the bumps were aggregates of unevenly scattered electron-dense material. The possibility that these inclusion bodies, originally thought to be Heinz bodies, represent α-globin aggregates is intriguing but remains to be proved.

In the course of performing experiments using hypertonic KCl to strip off more protein 3 from MS we observed that the level of protein 3 was increased in MS prepared from severe β-thalassemia patients (Table 3, Fig 2) but not in another hemoglobinopathy, sickle cell anemia. This increase in protein 3 was evident in both spx and non-spx thalassemia intermedia patients as well as transfusion dependent thalassemia major patients whose RBC are a mixed population. The lack of correlation between globin content and protein 3 content of the MS indicates that these are probably unrelated phenomena and that globin aggregates do not serve to link most of the protein 3 to the MS. Attachment of an
RBC MEMBRANE SKELETON ABNORMALITIES

integral protein like protein 3 to the cytoskeleton could result in an increase in membrane rigidity. Chasis et al. have shown that the increased RBC rigidity produced by monoclonal antityglycophorin A antibody is associated with the attachment of glycophorin A to the MS. In fact, MS prepared from splenectomized β-thalassemic patients retain some glycophorin as indicated by PAS staining. Recent data suggest that β-thalassemic protein 4.1 has abnormal structure and function. The retention of integral membrane proteins on the thalassemic skeleton might therefore be a consequence of abnormal interaction of protein 4.1 with both protein 3 and some of the glycophorins.

These studies are the first to explore the MS and IOVs of severely affected patients with β-thalassemia and show abnormalities in membrane skeletal assembly of their RBC. One of these changes, the increase in globin association to MS, is most clearly seen in spx thalassemic patients, thereby suggesting that this change may define in part the most severely affected cohort of cells that are preferentially removed by the splenic macrophages. However, the increase in protein 3 and glycophorins in MS is a common finding in all severe β-thalassemic MS studied and may represent a mechanism that could serve to produce RBC rigidity. These abnormalities may be a consequence of the hemichrome-mediated oxidative potential described in thalassemics. Further experiments will be needed to test these and other hypotheses.

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Erythrocyte membrane skeleton abnormalities in severe beta-thalassemia

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