Isolation and Partial Characterization of a High Molecular Weight Red Cell Membrane Protein Complex Normally Removed by the Spleen

By Samuel E. Lux and Kathryn M. John

Red cell membranes from all splenectomized and some functionally asplenic persons contained a small proportion of a very high molecular weight, sodium dodecyl sulfate (SDS)-insoluble protein aggregate not found in normals. This complex averaged 3.6% ± 1.7% (SD) of the total red cell membrane protein (range 1.2%-6.3%). It was identified in the marrow reticulocytes, but not the circulating red cells, of a patient with a normal spleen. In splenectomized individuals it was present equally in young and old circulating erythrocytes. When ghosts were disrupted and banded in a sucrose density gradient, the high molecular weight protein complex remained with the membrane fraction, indicating that it was not a particulate cytoplasmic contaminant. It was accessible to iodination by lactoperoxidase only at the inner membrane surface. The complex was remarkably resistant to dissociation. It was partially disaggregated by reduction in SDS with the release of the membrane protein, spectrin. Judging from its amino acid composition, however, the complex must contain one or more additional proteins which remain to be identified. These studies suggest that reticulocyte membranes contain a high molecular weight aggregate of spectrin and other membrane proteins and that splenic “surface remodeling” includes the removal of this protein complex as well as membrane lipids. The complex may prove to be a useful tool for further study of this poorly understood splenic function and for clinical assessment of splenic hypofunction.

IN SPLENECTOMIZED PERSONS many of the red cells are targeted, misshapen, or burdened with useless inclusions. These variations illustrate, in part, the ability of the spleen to remove or repair defective red cells. This capacity is sometimes subdivided into the “culling,” “pitting,” and “surface remodeling” functions of the spleen. Relatively little is known of these functions. “Culling” describes the ability of the spleen to remove and destroy red cells that are malformed, agglutinated, indeformable, or coated with antibodies or complement components. “Pitting,” on the other hand, is a restorative function. Useless or potentially harmful inclusions such as Heinz or Howell-Jolly bodies, siderotic granules, endocytic vacuoles, and intracellular parasites are removed (pitted) with little harm to the red cell other than loss of some of its membrane. The means by which the spleen accomplishes this surveillance and repair are imperfectly understood. For red cells coated with complement or

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IgG subtypes 1 and 3, specific receptors on phagocytes promote selection of the abnormal cells. In other cases the cell may be too indeformable or the inclusions too large to permit passage through the narrow fenestrations separating the splenic cords and sinuses. In most instances, however, the reason a particular red cell is culled or an inclusion pitted is unknown.

Even less is known of “surface remodeling.” This term refers to the postulated ability of the spleen to remove surplus membrane components from red cells. It is generally used with reference to the loss of membrane that accompanies maturation of reticulocytes during their first several days in the circulation. The principal evidence for this function derives from the old observation that target cells accumulate following splenectomy. Target cells are osmotically resistant, a characteristic of red cells with an abnormally high surface-to-volume ratio. By deduction, the spleen must normally remove surplus membrane from such cells. Shattil and Cooper and Come et al. have shown, in rats, that the stress reticulocytes induced by acute blood loss or hemolysis lose approximately one-third of their plasma membrane lipids during maturation. The membrane lipid is removed with comparatively little loss of cytoplasmic contents. It is presumed, but unproven, that a proportionate loss of membrane proteins occurs. This reticulocyte membrane maturation is at least partially attributable to the spleen since splenectomy decreases the loss of membrane lipid from stress reticulocytes by more than 50%. Normal reticulocytes also undergo some surface remodeling, which is mediated, in part, by the spleen. Compared to the stress reticulocyte, however, the remodeling process is a minor one.

The present studies evolved from an examination of the effect of splenectomy on red cell membrane proteins. They demonstrate that, in addition to membrane lipids, a very high molecular weight membrane protein complex is removed from red cells soon after their entry into the circulation, and that the presence of this complex is a characteristic of the asplenic state.

MATERIALS AND METHODS

Preparation of Membranes

Blood was collected in acid–citrate–dextrose solution and processed within 24 hr of collection. Red cell membranes were prepared by a modification of the method of Dodge et al. Erythrocytes were washed three times (4500 g-min*) at 4°C with 5 volumes of 310 mOsm sodium phosphate buffer, pH 7.4, and the supernatant and buffy coat were discarded. The washed, packed red cells were hemolyzed with 10 volumes of cold 20 mOsm sodium phosphate buffer, pH 7.4, collected by centrifugation (250,000 g-min), and washed five times with the same buffer. Under these conditions the membranes were a pale opalescent pink color and the supernatant contained only traces of hemoglobin. Care was taken to remove the pellet of unlysed leukocytes after each wash, to keep the membranes on ice, and to work as rapidly as possible in order to minimize proteolysis. Membranes were frozen immediately after preparation and stored in liquid nitrogen. In experiments where intact ghosts were necessary, the membranes were utilized immediately after preparation.

Polyacrylamide Gel Electrophoresis

Membrane proteins were analyzed by polyacrylamide gel electrophoresis in 0.2% sodium dodecyl sulfate (SDS) using the procedure of Fairbanks et al. as modified by Steck. Gels were 6 x 80

*g-min (force x time) allows conditions to be calculated for any given rotor.
mm and contained 3%–5% acrylamide. The N,N'-methylenebisacrylamide:acrylamide ratio was maintained at 0.0375:1 (w:w). The gels were stained with Coomassie brilliant blue (Sigma) as described by Weber and Osborn.\textsuperscript{14}

**Gel Filtration Chromatography in SDS**

Red cell membranes (10 mg) were removed from storage, thawed, and concentrated by centrifugation. The membrane pellet was suspended in 1 ml of 1% SDS, 10% sucrose, 0.01 M Tris-HCl, pH 7.8, and sufficient solid SDS was added to bring the final concentration to 4%. The solubilized membranes were layered onto a 1.2 x 45 cm column of Sepharose-2B and eluted at 25°C with 1% SDS, 0.01 M Tris-HCl, pH 7.8, at a flow rate of 3-4 ml/hr. Absorbance of the eluate was monitored at 280 nm. Because the eluting buffer slowly extracted an unknown ultraviolet-absorbing substance(s) from all types of tubing tested, it was necessary to use a glass buffer reservoir and to flush the column outlet tubing for 15-30 min prior to loading in order to maintain a low background on the chromatograms. After 2-5 successive runs each column had to be repoured because of packing.

**Density Gradient Centrifugation**

Red cell membranes (15 mg) suspended in isotonic sodium phosphate buffer, pH 7.4, were frozen and thawed six times, and disruption of the membranes was confirmed by phase microscopy. The membrane suspensions were layered onto linear sucrose density gradients (13 ml, d 1.15 - 1.21 g/ml) and centrifuged at 39,000 rpm for 16 hr (1.9 x 10⁹ g-min) in a Spinco-SW4O rotor at 4°C. The gradients were harvested with an Isco density gradient fractionator into 0.5-ml fractions and were analyzed by absorbance at 280 nm.

**Iodination of Erythrocyte Membranes**

Intact erythrocytes, resealed ghosts, and unsealed ghosts were iodinated with \(^{125}\text{I}\) using lactoperoxidase and a peroxide-generating system containing glucose and glucose oxidase.

**Intact erythrocytes.** Freshly drawn red cells were washed three times in phosphate-buffered saline (PBS; 1 volume 310 mOsm sodium phosphate buffer, pH 7.4, and 19 volumes 0.15 M NaCl). Red cells (6 x 10¹⁰) were suspended in 10 ml of the same buffer and KI (final concentration 0.001 mM), glucose (final concentration 11 mM), lactoperoxidase (4.8 units), and carrier-free Na\(^{125}\text{I}\) (10-100 μCi) were added. Iodination was initiated by the addition of glucose oxidase (1.4 units), and the samples were incubated at room temperature for 30 min. The reaction was terminated with 20 ml of cold PBS, and the erythrocytes were collected by centrifugation and washed five times with 6 volumes of the same buffer to remove excess reactants. Membranes were prepared from the labeled erythrocytes by the standard procedure.

**Unsealed ghosts.** Washed red cells (6 x 10¹⁰) were hemolyzed as above, resuspended in 10 ml of PBS, and resealed by incubation for 5 min at 0°C and 30 min at 37°C. The resealed ghosts were cooled to room temperature and iodinated as above. The reaction was terminated with 20 ml of cold 20 mOsm buffer, and the ghosts were washed five times with 6 volumes of the same buffer.

**Resealed ghosts.** Washed red cells (6 x 10¹⁰) were hemolyzed as above, resuspended in 10 ml of PBS, and resealed by incubation for 5 min at 0°C and 30 min at 37°C. The resealed ghosts were cooled to room temperature and iodinated as above. The reaction was stopped with 20 ml of cold PBS, and the ghosts were washed three times with 6 volumes of PBS and then five times with 6 volumes of 20 mOsm sodium phosphate buffer, pH 7.4.

**Production of Ghosts Containing Endocytic Vesicles**

Washed red cells were suspended in 10 volumes of a buffer containing 130 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1.5 mM CaCl₂, 1 mM MgCl₂, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 20 mM hydrocortisone succinate\textsuperscript{15} (Sigma), pH 7.4, and were incubated at 37°C for 2 hr. Phase contrast microscopy of the incubated red cells showed extensive endocytosis. This finding was confirmed by assay of acetylcholinesterase, an enzyme located on the outer membrane surface,\textsuperscript{16} and by osmotic fragility tests. The acetylcholinesterase assay indicated that 20% of the membrane surface had become inaccessible during the incubation.\textsuperscript{17} Osmotic fragility curves showed that the entire erythrocyte population became more osmotically fragile.
after the incubation, indicating that all of the erythrocytes were forming endocytic vesicles. Ghosts were prepared from the incubated red cells and, judging by phase microscopy, each contained 1-4 vesicles. These ghosts were dissolved in 4% SDS and chromatographed on Sepharose-2B in SDS as described earlier.

Other Procedures

Protein concentrations were determined by the method of Lowry et al. or by amino acid analysis. Amino acid analyses were performed as previously described. Ultraviolet spectra were determined on a Cary 118 instrument.

RESULTS

Detection and Quantitation of High Molecular Weight Complex

Erythrocyte membrane proteins from splenectomized and nonsplenectomized individuals were initially compared by polyacrylamide gel electrophoresis (PAGE) in SDS (SDS-PAGE) (Fig. 1). The gel patterns obtained were similar to those previously reported. The bands were numbered according to the system instituted by Fairbanks et al. and extended by Steck. The identity of the bands was established by their relative mobility and by their molecular weights. In addition, the bands designated 5 and 6 were shown to be extractable with low and high ionic strengths, respectively. Several additional bands, apparent in our gels (bands 4.3, 4.4, 4.5, and 8) but not designated by Fairbanks et al. or Steck, were assigned numbers in sequence.

Splenectomy induced no evident alteration in the polyacrylamide gel patterns whether stained for protein with Coomassie blue (Fig. 1) or for glycoprotein with a periodic acid-Schiff stain (not shown).

Since very high molecular weight proteins or protein complexes might not enter 5% polyacrylamide gels, membranes from splenectomized and non-
Fig. 2. Gel filtration chromatography on Sepharose-2B in SDS. Erythrocyte membranes (10 mg) from a normal (A) or splenectomized (B) individual were dissolved in 2 ml of 4% SDS, 0.01 M Tris-HCl, pH 7.8, and chromatographed on a 1.2 x 45 cm column of Sepharose-2B. The column was eluted at 25°C with 1% SDS, 0.01 M Tris-HCl, pH 7.8, at 4 ml/hr. V₀: void volume.

Splenectomized individuals were also compared by SDS gel filtration chromatography in porous (2% agarose) gels. With this technique red cell membranes from nonsplenectomized individuals emerged in a single asymmetric peak (Fig. 2A). Membranes from splenectomized persons, however, contained additional material which was larger than any of the normal membrane proteins and emerged in the void volume (Fig. 2B), indicating that its apparent molecular weight in SDS was 40 million daltons or more. The peak was present in freshly prepared membranes as well as in those stored in liquid nitrogen. Its proportion was not altered by repeatedly freezing and thawing the membranes before solubilization and chromatography. No ultraviolet-absorbing fractions were detected when solubilizing buffer alone was run. The proportion of the high molecular weight peak in repetitive runs was reproducible to ±4%.

As shown later, amino acid analyses and ultraviolet absorption spectra indicated that the void volume material contained protein. Since almost all proteins are dissociated into their constituent polypeptide subunits in SDS\textsuperscript{14} and the largest known polypeptides are nearly two orders of magnitude smaller than this material, we concluded that it was an aggregate containing one or more red cell proteins.

Survey for the High Molecular Weight Peak

This protein complex was present in each of 10 different splenectomized individuals and in none of 10 persons with intact, functioning spleens (Table 1). It averaged 3.6% ± 1.7% of the total red cell membrane, with a range of 1.2%–6.3%. There was no definite relationship between the proportion of the high molecular weight protein material and the nature of the underlying disease.

In one patient, who was splenectomized for staging of Hodgkin disease, the protein complex was not detectable in the first weeks postoperatively, but gradually increased over the ensuing 4 mo.
Table 1. Proportion of High Molecular Weight Membrane in Various States

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Patients</th>
<th>High Molecular Weight Peak* (Percent of Total Membrane Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Splenectomized</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>5</td>
<td>2.3–6.3</td>
</tr>
<tr>
<td>Congenital asplenia</td>
<td>1</td>
<td>2.4</td>
</tr>
<tr>
<td>Trauma</td>
<td>1</td>
<td>3.0</td>
</tr>
<tr>
<td>Hodgkin disease</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>Idiopathic thrombocytopenic purpura</td>
<td>2</td>
<td>2.1, 3.8</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td><strong>3.6 ± 1.7 (SD)</strong></td>
</tr>
<tr>
<td><strong>Unsplenectomized</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>6</td>
<td>0.0</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>Hodgkin disease</td>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Sickle Cell Anemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 yr old</td>
<td>1</td>
<td>0.0</td>
</tr>
<tr>
<td>7 yr old</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>15 yr old</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>21 yr old</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Cord blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full term, normal infants</td>
<td>2</td>
<td>0.0, 0.5</td>
</tr>
</tbody>
</table>

*Ten milligrams of erythrocyte membranes from each individual were chromatographed on a column of Sepharose-2B in SDS as described in Fig. 2. The proportion of the high molecular weight peak is expressed as (area of void volume peak/total area of all membrane peaks) × 100. Areas were determined by weighing tracings of the chromatograms. In chromatograms in which the absorbance tracing did not return to the baseline between the void volume peak and the main membrane protein peak, the area of the void volume was defined by a perpendicular dropped from the nadir between the two peaks.

Since newborn infants and patients with sickle cell anemia have functional hyposplenism by some criteria, we also examined cord blood and sickle erythrocyte membranes for the presence of this protein material. In four patients with sickle cell anemia, the presence of the protein complex was correlated with age (Table 1) and roughly approximated the time course of autosplenectomy due to infarction and scarring that occurs in that disease. A small amount of the complex was detected in one of the two cord blood specimens examined (Table 1).

These observations confirm that the presence of this aggregate in red cell membranes is due to the absence of the spleen and indicate that this anomaly is not an early or especially sensitive indicator of the hyposplenic state, at least under the chromatographic conditions employed here.

**Origin of the High Molecular Weight Protein Complex**

To test whether the protein complex is a product of cellular aging, young and old red cell fractions were isolated by repetitive ultracentrifugation of erythrocytes from an 18-yr-old girl splenectomized for idiopathic thrombocytopenic purpura (Table 2). Reticulocyte counts indicated a 40-fold relative enrichment of reticulocytes in the younger, less dense red cells, compared to the more dense, older erythrocytes. However, the proportion of the high molecular weight protein complex was similar in the young and old red cells. These findings suggest that the complex is not a product of cellular aging but rather is due to the absence of the spleen.
Table 2. Proportion of High Molecular Weight Membrane Peak in Young and Old Red Cells From a Splenectomized Patient

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Reticulocytes (%)</th>
<th>High Molecular Weight Peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>4.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Old</td>
<td>0.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Splenectomized for idiopathic thrombocytopenic purpura. Similar results were obtained in an identical experiment with another patient.

†Erythrocytes were washed three times with isotonic phosphate buffer, pH 7.4, and the buffy coat was removed. The cells were resuspended to a hematocrit of 85% in isotonic phosphate buffer and centrifuged in a fixed-angle head rotor (15,000 g, 60 min, 37°C). The erythrocytes in the top and bottom 3% of the cell column were removed and designated as the “young” and “old” cell fractions, respectively. Membranes were prepared from these cells and chromatographed on Sepharose-2B in SDS as described in Fig. 2.

weight peak was the same in each fraction. This suggested that either red cells emerged from the bone marrow bearing the protein complex or acquired it soon after entering the circulation and, in the absence of the spleen, retained it throughout their life span.

To distinguish between these alternatives, 15 ml of bone marrow were obtained from a normal nonsplenectomized patient, and the nonnucleated red cell fraction was isolated by differential centrifugation. As anticipated, this fraction exhibited a high molecular weight component on SDS gel chromatography (Fig. 3) that was not detected on chromatography of an equal amount

Fig. 3. Gel filtration chromatography of membranes from marrow erythrocytes. Fifteen milliliters of bone marrow were obtained from a nonsplenectomized patient with acute lymphocytic leukemia in remission. The specimen was microscopically normal and contained abundant erythroid precursors. Marrow reticulocytes were separated from the nucleated marrow elements by differential centrifugation. No attempt was made to determine the extent to which the marrow fraction was diluted with circulating erythrocytes. Ten milligrams of membranes from this nonnucleated red cell fraction were chromatographed on Sepharose-2B in SDS as described in Fig. 2.
of membranes from the patient's circulating red cells (not shown). It should be noted that the proportion of the high molecular weight peak (1.76%) in the bone marrow fraction represented a minimum estimate since the marrow red cells were probably diluted with unknown numbers of circulating erythrocytes during sampling.

Location of the High Molecular Weight Protein Complex

Because gel filtration chromatography indicated that the protein complex is very large, the question arose whether the complex is a true membrane constituent (i.e., physically attached to the membrane) or whether it is a cytoplasmic component which was so large that it could not escape from the ghost during hypotonic hemolysis. To test this question erythrocyte ghosts from a woman splenectomized for traumatic splenic rupture were frozen and thawed six times, and their disruption was monitored by phase contrast microscopy. The membrane fragments were centrifuged through a sucrose gradient (d 1.15–1.21 g/ml) and the distribution of protein in the gradient was analyzed by absorbance at 280 nm (Fig. 4A).

Two major (II and III) and two minor (I and IV) bands were detected. Erythrocyte membranes from a normal individual, though lacking the high molecular weight protein complex, produced an identical banding pattern (not shown). Band I was a sharp, reddish brown, turbid zone at d 1.155 g/ml. Band IV, at d 1.203 g/ml, was colorless and nonturbid. They were not examined

Fig. 4. Density gradient centrifugation of erythrocyte membranes. Fifteen milligrams of erythrocyte membranes from a woman splenectomized for splenic trauma were frozen and thawed six times, layered onto a linear sucrose gradient (13 ml, d 1.15–1.21 g/ml), and centrifuged at 4°C for 1.9 x 10^9 g-min in a swinging bucket rotor. (A) Following centrifugation the gradient was fractionated and analyzed for absorbance at 280 nm. (B) Fractions of peaks II and III denoted by the heavy bar in A were pooled and chromatographed on a column of Sephrose-2B in SDS. Conditions of chromatography were as described in Fig. 2.
further. Bands II and III were opaque, white zones which were clearly delineated in the gradient and microscopically resembled the starting frozen and thawed membrane sample. The difference(s) in these two fractions were not investigated in this study. Bands II and III were pooled and assayed for the high molecular weight complex by gel filtration chromatography in SDS (Figure 4B). All (115%) of the complex present in the initial membranes was recovered in these two bands and none was found at the bottom of the gradient, indicating that the high molecular weight protein complex was membrane associated and not a trapped cytoplasmic contaminant.

To determine whether the protein complex was located on the inside or outside surface of the membrane, intact red cells, unsealed ghosts, and resealed (see Materials and Methods) ghosts from the patient splenectomized for splenic trauma were labeled with $^{125}\text{I}$ using lactoperoxidase and a peroxide-generating system (glucose–glucose oxidase). In the presence of hydrogen peroxide, lactoperoxidase attaches iodine to accessible proteins. Only two of the many red cell membrane protein bands (glycophorin and band 3) are labeled when intact red cells or resealed ghosts are iodinated by lactoperoxidase,25 and both of these proteins are known to be exposed on the external membrane surface by other criteria.26,27 In contrast, in unsealed ghosts all of the membrane proteins are labeled,25 including some, such as bands 1 and 2 (spectrin) and band 6 (glyceraldehyde-3-phosphate dehydrogenase), which are known to be confined to the cytoplasmic membrane surface by other criteria.28-29

In the present study (Fig. 5) the $^{125}\text{I}$-labeled membranes were chromatographed on 2% agarose in SDS and the distribution of protein (open circles) and radioactivity (solid circles) was recorded. The expected anisotropy of labeling was confirmed by the observation that the shoulder on the leading

![Fig. 5. Gel filtration chromatography of red cell membranes from a splenectomized individual labeled on one or both surfaces with $^{125}\text{I}$. Lactoperoxidase, Na $^{125}\text{I}$, and a peroxide-generating system (glucose–glucose oxidase) were added to intact red cells (A), resealed ghosts (B), and unsealed ghosts (C). Details of the procedure are presented in Materials and Methods. Labeled membranes were chromatographed on Sepharose-2B in SDS as described in Fig. 2 and analyzed for absorbance at 280 nm (○) and radioactivity (●). Note that the radioactivity scales vary for the different membrane preparations. S: shoulder on the leading edge of the main membrane protein peak.](http://www.bloodjournal.org/content/77/6/633)
Table 3. Amino Acid Analysis of Membrane Protein Fractions

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>High Molecular Weight Peak From Asplenic Patient</th>
<th>Unfractionated Erythrocyte Membrane Proteins (mole/100 moles amino acids)</th>
<th>High Molecular Weight Peak From Ca ++ treated Ghosts*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient Spectrin</td>
<td>Membrane Proteinst</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.7</td>
<td>11.0</td>
<td>8.2-9.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.8</td>
<td>4.4</td>
<td>5.7-5.9</td>
</tr>
<tr>
<td>Serine</td>
<td>9.7</td>
<td>5.3</td>
<td>6.3-8.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>12.9</td>
<td>18.8</td>
<td>12.1-12.9</td>
</tr>
<tr>
<td>Proline</td>
<td>3.8</td>
<td>2.1</td>
<td>4.3-5.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>10.4</td>
<td>5.1</td>
<td>6.6-7.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.1</td>
<td>9.2</td>
<td>7.3-8.2</td>
</tr>
<tr>
<td>Valine</td>
<td>5.7</td>
<td>4.7</td>
<td>5.8-7.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.9</td>
<td>1.5</td>
<td>1.1-2.4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.5</td>
<td>3.9</td>
<td>4.4-5.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.4</td>
<td>12.4</td>
<td>11.3-12.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.7</td>
<td>2.2</td>
<td>1.8-2.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0</td>
<td>3.3</td>
<td>3.6-4.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.6</td>
<td>7.1</td>
<td>4.8-5.2</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.7</td>
<td>3.0</td>
<td>2.3-2.4</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.4</td>
<td>6.1</td>
<td>4.5-4.8</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>—</td>
<td>—</td>
<td>0.9-1.4</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>—</td>
<td>—</td>
<td>0.4-2.5</td>
</tr>
</tbody>
</table>

*No correction was made for possible degradation or incomplete hydrolysis of particular residues.
†The values for spectrin and the high molecular weight peaks are the average of two different preparations of each protein.
‡Data from Bakeman and Wasemiller31 and Rosenberg and Guidotti.32
§Data from Carraway et al.33
|| These residues were completely destroyed by the hydrolysis conditions used [6 N HCl, 1:2000 (v,v) 2-mercaptoethanol, 24 hr, 100°C].19

edge of the absorbance peak (designated by the S in Fig. 5A) was only labeled in the unsealed ghosts (Fig. 5C). This shoulder contained primarily the high molecular weight spectrin bands (bands 1 and 2; Lux and John: Unpublished observations) which are confined to the cytoplasmic surface.28 Similarly, the high molecular weight peak was only labeled in the unsealed ghosts (Fig. 5C), indicating that it too was only exposed on the inner face of the red cell membrane.

Characterization of the High Molecular Weight Fraction

The isolated high molecular weight complex in SDS (0.1%) was colorless and slightly turbid and formed irregular fibrils when dialyzed against water. Amino acid analyses of this complex (Table 3) proved that it was protein. The amino acid composition differed significantly from some of the other proteins located or exposed on the inside surface of the red cell membrane, including spectrin (Table 3), glycophorin,34 glyceraldehyde-3-phosphate dehydrogenase (Band 6),35 rabbit muscle actin,36 and hemoglobin (including either α or β chains),38 as

It is noteworthy that standard protein assays such as the Lowry et al.18 or biuret30 methods substantially underestimated the protein content determined by amino acid analysis, presumably because the reaction conditions failed to disaggregate the complex, limiting the color development.
†The amino acid composition of erythrocyte membrane actin (Band 5)37 has not been reported, but actins, in general, are closely related structurally.
well as unfractionated erythrocyte membrane protein (Table 3). The amino acid composition of the high molecular weight complex is similar to the composition of calf brain tubulin, but the significance of this is uncertain. To date tubulin has not been identified in erythrocytes and we have not pursued this observation further.

The ultraviolet spectrum (not shown) of the peak material was also compatible with a protein identity. The absorbance band at 265 nm characteristic of nucleic acids was not evident.

Under certain conditions some of the red cell membrane proteins located on the cytoplasmic membrane surface can be selectively extracted. Bands 1, 2, and 5 are solubilized by low ionic strength solutions of EDTA; band 6 is removed by treatment of ghosts with isotonic saline; and bands 1, 2, 4, 1, 4, 2, 5, and 6 are extracted with 0.1 M sodium hydroxide. However, the high molecular weight protein complex has not been extracted from ghosts by treatment with either 0.001 M sodium EDTA, pH 9 (48 hr at 4°C); 0.15 M NaCl (48 hr at 4°C); or 0.1 M NaOH (1 hr at 4°C).

The isolated high molecular weight complex would not enter a low porosity (3%, acrylamide) SDS gel even if it were pretreated by exhaustive succinylation or by heating (100°C, 10 min) in 2%, SDS. When the aggregated complex in 2%, SDS was reduced with 100 mM dithiothreitol prior to electrophoresis, two bands with the mobility and spacing of spectrin appeared in the gel (Fig. 6). These bands were not present in samples of the complex pretreated with 2%, SDS without dithiothreitol. Reduction of the spectrin standard did not alter its gel electrophoretic pattern. Even with vigorous reduction only a small pro-

Fig. 6. Polyacrylamide gel electrophoresis in SDS of spectrin and the reduced high molecular weight (V₀) peak. The spectrin standard contained traces of band 5. The protein fractions (15 µg) were incubated at 37°C in 2% SDS, 10 mM Tris–HCl, 1 mM EDTA, 145 mM sucrose, 100 mM dithiothreitol, pH 7.4, for 1 hr, electrophoresed in 5% acrylamide gels, and stained with Coomassie blue. Two protein bands corresponding to spectrin in mobility and spacing were present in the reduced high molecular weight peak but were not seen in an identical aliquot treated without reduction (not shown).
portion of the complex could be disaggregated (roughly 10% of the total protein in the complex), so it was impossible to determine how much of the high molecular weight material was spectrin. Differences in the amino acid analyses of the two proteins (Table 3), however, indicated that the high molecular weight complex must contain at least one other protein component.

Examination of Ghosts Containing Endocytic Vesicles

We wondered if the high molecular weight complex could be related to the endocytic vesicles that are present in many circulating red cells after splenectomy.42 These vesicles are rare in persons with functioning spleens,42 so, by inference, the spleen must either remove them or inhibit their formation. Although many of these vesicles would have separated from the membrane,42 they would probably band with plasma membrane fragments on a sucrose gradient and would presumably contain spectrin. To test this possibility we induced red cell endocytosis with hydrocortisone15 and prepared ghosts containing endocytic vesicles from these erythrocytes. Acetylcholinesterase assays indicated that 20% of the plasma membrane was internalized.17 However, SDS gel chromatography showed no detectable high molecular weight complex.

DISCUSSION

In these experiments it was found that the red cells of all the splenectomized and some functionally asplenic persons contained a small proportion (1%-6%) of a very large, membrane-bound protein aggregate which was present in equal amounts in both young and old red cells. In normal individuals a similar high molecular weight protein was found in nonnucleated marrow red cells but was absent from circulating erythrocytes. This finding suggests that the protein aggregate is associated with the membranes of reticulocytes as they enter the circulation and is normally removed by the spleen during or soon after reticulocyte maturation, perhaps in a manner analogous to the removal of lipids from the red cell membrane during the same period. When the spleen is absent or nonfunctional the protein complex remains attached to the red cell membrane throughout the life span of the cell. Since the survival of normal red cells is not decreased following splenectomy,43,44 the presence of this large protein complex must not be especially harmful to the cell.

The identity of this material remains enigmatic, due largely to its remarkable resistance to dissociation in SDS. This property alone distinguishes the complex from almost all other proteins, including most potential "contaminants" (see below) and suggests that it might be held together by covalent bonds instead of the usual noncovalent ones. Since some spectrin can be solubilized from the complex after reduction but not before, disulfide bonds must contribute to the organization of the complex. Since the complex cannot be completely dissociated by prolonged, vigorous reduction in the presence of SDS, however, other types of covalent bonds45,47 may also be important. The complex was incompletely dissociated in our experiments, so it is impossible at present to determine whether spectrin is a major constituent or only a minor one. As noted in Results, differences in the amino acid composition of the two proteins indicate that the high molecular weight complex must contain at least
One nonspectrin protein component. Its exact definition will have to await discovery of more effective means of dissolution or analysis.

One question that troubled us initially was whether the high molecular weight complex was simply the chemical residue of one of the well-known morphologic inclusions that accumulate in red cells after splenectomy. These include nuclear remnants (Howell-Jolly bodies), denatured globin chains (Heinz bodies), ferritin aggregates (siderosomes), endocytic vesicles, and various cytoplasmic organelles such as mitochondria or ribosomes. Two observations indicate that the high molecular weight complex is membrane bound: it remains with the two major membrane bands when fragmented red cell membranes are banded on a sucrose density gradient (Fig. 4), and it contains spectrin, a major red cell membrane component (Fig. 6).

Of the morphologic inclusions above, only Heinz bodies are usually considered to be bound to the plasma membrane of the red cell. The high molecular weight complex differs from Heinz bodies in two important respects: (1) Heinz bodies are denatured globin chains. The amino acid composition of the high molecular weight complex is not compatible with either α or β globin chains in many respects; particularly since it contains substantial amounts of isoleucine (2.5 moles/dl amino acids), whereas hemoglobin A contains none. (2) In our own (unpublished observations) and others' experience Heinz bodies dissociate into a variety of lower molecular weight complexes, including monomeric globin chains, when they are reduced and dissolved in SDS, while the high molecular weight complex is extremely resistant to dissolution under the same conditions.

It also seems unlikely that the high molecular weight complex corresponds to any of the other morphologic inclusions listed above. In addition to its membrane location, the high molecular weight complex lacks the characteristic nucleic acid ultraviolet absorption band that would be present in nuclear remnants or polyribosomes and differs from ferritin in color, dissociation in SDS, and amino acid content. Furthermore, with the exception of endocytic vesicles, the presence of spectrin in any of these inclusions would be most surprising. As noted in Results, endocytic vesicles would likely band with plasma membrane fragments on a sucrose gradient and would presumably contain spectrin. It seems unlikely, however, that endocytic vesicles would be insoluble in SDS and, in fact, when extensive endocytosis was induced in vitro by incubation of normal red cells with hydrocortisone, no high molecular weight complex was formed.

We know of no other report of SDS-insoluble membrane components from fresh, unperturbed erythrocytes. SDS-insoluble membrane protein complexes, however, can be induced in erythrocytes or ghosts under certain circumstances. We have observed such complexes in normal erythrocytes after ATP stores are exhausted, presumably from oxidation of membrane proteins. Palek and his co-workers have made similar observations. High molecular weight membrane protein aggregates are also formed in erythrocyte ghosts exposed to relatively high concentrations of calcium ion (0.1–5 mM). The relationship of these aggregates to each other and to the present complex is unclear. The complex we detected in ghosts of metabolically depleted erythrocytes was excluded from Sepharose-2B in SDS, like the present complex, but it had a different amino
acid composition and was much more easily and completely dissociated by reduction in SDS. It contained hemoglobin and several other membrane proteins, including spectrin, but the proportion of spectrin was relatively smaller.

Palek et al. have also identified reducible membrane protein aggregates in ATP-depleted erythrocyte membranes, but they observed a larger proportion of spectrin as well as actin, band 3, and an unidentified 47,000-dalton component. These investigators fractionated their membranes by SDS-PAGE rather than by agarose-gel chromatography in SDS. Consequently, they analyzed complexes of 0.26-1.0 $\times 10^6$ daltons, while we examined a complex with a molecular weight greater than 40 $\times 10^6$. This finding suggests that different kinds of membrane protein complexes form in ATP-depleted erythrocytes and that the exact composition of a particular complex may depend on a number of factors, including size and, probably, the extent of metabolic depletion. Consequently, it is difficult to argue that the high molecular weight complex observed in asplenic patients is not related to the complex(es) seen in ATP-depleted erythrocytes simply because it differs in composition. The differences in dissociation, however, are difficult to reconcile.

The membrane protein aggregates from ATP-depleted erythrocytes are easily dissociated by reduction, which indicates that they are largely maintained by disulfide cross links. Under the same conditions only a small amount of spectrin is released from the asplenic complex, which suggests a different kind of intermolecular cross-linking. Furthermore, if the asplenic complex is the result of ATP depletion or other events related to ATP depletion (e.g., oxidative damage to membrane proteins), this damage would probably be cumulative and the complex would be more evident in older erythrocytes. Instead it was present in equal proportions in reticulocytes and aging erythrocytes (Table 2).

Palek and his associates found they could duplicate the effects of metabolic depletion by incubating unsealed fresh ghosts in 0.1 mM calcium. This amount is about 5-10 times the calcium concentration of the normal erythrocyte but is within the range seen in pathologic situations. Under these conditions small dissociated aggregates are formed.52 With higher concentrations of calcium (1-5 mM) a large aggregate is produced33 which resembles the aggregate found in asplenic patients in size and resistance to dissociation and is very similar, though not identical, in amino acid composition (Table 3). Like the asplenic complex this calcium-induced membrane protein aggregate contains spectrin and at least one other protein. Despite the resemblance it is difficult to imagine that calcium could be responsible for the membrane protein complex reported here since the calcium concentrations required to form an undisassociated membrane protein aggregate in vitro far exceed normal erythrocyte calcium levels.

The identification of this complex could be technically useful in two situations. First, since removal of the high molecular weight complex is the most objective evidence of the interaction between red cells and the spleen that we refer to as membrane "remodeling," it should be a useful model for further study of this poorly understood splenic function. Second, since it has been reproducibly present in asplenic patients it serves as a marker for the asplenic state. More important, it may prove useful as an indicator of a poorly functioning spleen. Though other indicators of hyposplenism, such as Howell-Jolly bodies, are
more easily detected than the high molecular weight complex, we do not know whether all the various functions of the spleen are equally depressed in situations in which splenic function is abnormal. Since splenic hypofunction occurs in some relatively common conditions (e.g., prematurity, sickle cell disease, and high-dose corticosteroid therapy) and may well contribute to the increased risk of sepsis in these conditions, it will be important to determine the relative usefulness of various tests in detecting clinically important degrees of splenic hypofunction.

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Isolation and partial characterization of a high molecular weight red cell membrane protein complex normally removed by the spleen

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