Lipid Membrane Peroxidation in β-Thalassemia Major

By E. A. Rachmilewitz, B. H. Lubin, and S. B. Shohet

The composition of membrane lipids was studied in 17 splenectomized and eight unsplenectomized patients with β-thalassemia major and compared to normal controls. The results showed a nearly twofold increase in total cell lipids; a reduction in the percentage, but not the absolute amount of phosphatidylethanolamine, and a corresponding increase in phosphatidylcholine in the lipids; a considerable increase in the percentage of the saturated fatty acid, palmitic acid, and a reciprocal decrease in the polyunsaturated fatty acid, arachidonic acid; a twofold increase in the amount of malondialdehyde (MDA) generated after peroxide threat to the RBC when calculated either per gram hemoglobin or per cell; no change in the amount of MDA generated when calculated per microgram of membrane phosphorus at risk per cell; and a considerable decrease in serum α-tocopherol (vitamin E) levels. Thalassemic erythrocytes contain more lipid per cell which is susceptible to peroxidation. In addition, the distribution of fatty acids in these cells suggests that autooxidation of that lipid may have occurred. Autooxidation may be initiated by free radicals, which are constantly formed in the normal red cell, and may be especially prevalent when unstable hemoglobins are present. The low MCHC or some other intracellular defect of thalassemic cells may allow such potent oxidants to find their way to the cell membrane. Vitamin E, a biologic antioxidant is decreased in these patients, and clinical supplementation may be indicated to prevent some of the membrane damage in thalassemia.

The peripheral red blood cells (RBC) of patients with β-thalassemia major show virtually every kind of morphologic abnormality that has been described. This observation in itself suggests that the membrane of the thalassemic red cell is severely damaged. A more detailed morphologic description of the membrane changes has been obtained by ultrastructural studies of circulating thalassemic RBCs. Indentations and unfolding of the plasma membrane with marked vacuole formation, together with many bizarre membrane forms and myelin figures, have been observed. In addition, Stocks et al. have found an increased susceptibility to exogenous peroxidant threat in thalassemic RBCs, further implying a biochemical defect in the membrane. However, the biochemical changes in the thalassemic membrane corresponding to the increased peroxide susceptibility and the morphologic defects in these short-lived cells are not fully understood.

In the current studies, we have approached this problem by an examination of red cell lipid content and composition, together with measurements of lipid peroxidation products after peroxide exposure. In addition, we have obtained
measurements of serum $\alpha$-tocopherol and carotene levels for correlative purposes.

We have confirmed the increased susceptibility of thalassemia RBC to peroxide threat, and have found that the extent of peroxidant damage is both closely related to increased amounts of susceptible lipid within each RBC and consistent with free radical-mediated attack upon this lipid. We suggest that this increased lipid "at risk," which may be the chemical counterpart of previously observed electron micrographic changes, is the basis of the increased $H_2O_2$ susceptibility of these cells in vitro, and that it may be related to their short survival in vivo as well.

MATERIALS AND METHODS

Fresh anticoagulated (ACD) blood was obtained from 25 patients with $\beta$-thalassemia major, two normal adults as "shipment controls" in Jerusalem (A.S. and E.R.), four nonthalassemic splenectomized controls, and a further control group of 25 normal adults in San Francisco. The majority (20) of the thalassemic patients were of Kurdish Jewish extraction, four were American-Italians, and one was an Israeli Arab. The pertinent hematologic data and hemoglobin analyses of most of these patients have been published elsewhere.4

Since the majority of the thalassemic patients required intermittent blood transfusions, the blood for the various studies was collected as late as possible after preceding transfusions. (The number of days post-transfusion are enumerated in Table I.) With the exception of the Italian-American samples which were determined directly, the various blood samples, including the "shipment controls," were kept at 4°C and shipped by air directly from Jerusalem to San Francisco. The normal controls (E.R. and A.S.) were examined on two separate occasions. Fresh peripheral blood smears were prepared in Jerusalem, and a reticulocyte count and differential count of 200 nucleated cells was carried out in each case. Following arrival in San Francisco, complete blood counts and reticulocyte counts were again performed on each sample according to standard procedures.5 In the 30 hr of transport, the reticulocyte counts did not change appreciably. The red cells were then separated from the plasma and residual leukocytes and platelets with multiple washings in Krebs-Henseleit buffer at pH 7.4, as previously described.6 Following reconstitution to a hematocrit of 50%, the white blood cell count was again determined and found to be less than 100 cells per cu mm.

Lipid Analysis

Erythrocyte lipids were extracted and separated by a slight modification of Rose's method.7,8 Major lipid classes were isolated by thin layer chromatography on Silica Gel H plates* by Skipski's method.9 Separated lipids were identified by brief iodine staining and marked. After complete evaporation of the iodine, they were scraped from the plate, and determinations of lipid phosphorus were made on fractions of sonicated suspensions of this material by the direct method of Parker and Peterson.10 For the determination of lipid fatty acid composition, methylesters of the unseparated lipid extracts were prepared using boron trifluoride.11 The extracts were kept under nitrogen and protected against spontaneous oxidation with butylated hydroxytoluene.12 Gas liquid chromatography was performed by methods similar to those of Dodge and Phillips,13 using an 8-ft glass column packed with 7% EGSS-X-on Gas chrome P., 100-120 mesh, with a hydrogen flame ionization detector. The carrier gas flow rate was 30 ml/min, and the column temperature was maintained at 190°C and the injector at 215°C. Retention times were standardized by comparison with a known standard mixture of fatty acid methylesters.

Peak areas were calculated by triangulation and were corrected in proportion to the molecular weights of the component fatty acids. The butylated hydroxytoluene which was added as an antioxidant, emerged from the column with a retention time similar to that of methyl myristate, and thus myristic acid was not independently determined. The gas-liquid chromatography methods

*Brinkman Instruments, Inc., Westbury, N. Y.
were not capable of distinguishing the geometric isomers of unsaturated fatty acids or of separately resolving dimethyl-acetyl derivatives of plasmalogens.\textsuperscript{13}

**Lipid Auto-Oxidation**

The assay for susceptibility of membrane lipids to auto-oxidation was based on the generation under oxidative stress of malonyldialdehyde (MDA), a secondary breakdown product of lipid peroxides.\textsuperscript{14} The MDA assay was carried out according to the method of Stocks et al.\textsuperscript{3}

Serum tocopherol (vitamin E) and β-carotene levels were performed according to the method of Hashim and Schuttringer\textsuperscript{15} and Quaif et al., respectively.\textsuperscript{16}

**RESULTS**

**Hematologic Data**

The mean reticulocyte count in 20 patients with β-thalassemia major was 4.8\%, while the mean in the normal adults was 0.25\% (Table 1). The mean nucleated cells in 17 patients who were splenectomized was 80,300/cu mm (Table 1). Differential counts showed that between 80\% and 90\% of these nucleated cells were normoblasts, confirming results of a previous study,\textsuperscript{2} and the rest were white blood cells. In nonsplenectomized thalassemic patients, the mean nucleated count was 17,200/cu mm, and the percentage of normoblasts was between 0\% and 20\%. Expectedly, in the controls, all nucleated cells were from the white cell series.

The average time after the last transfusion, before the blood was taken for the present studies, was 71 days, indicating that approximately two-thirds of the donor blood had been eliminated from the circulation. The approximate number of total transfusions, which was estimated from patients' records and recorded separately for each patient in Table 1, varied from 10 to 150.

**Lipid Analyses**

Several differences were found when the lipid analyses of the thalassemic RBCs were compared with the control cells.

(1) The total quantity of lipid phosphorus was nearly twice that of both the "shipment" controls ($p < 0.01$, Table 1) and the average of 25 normal controls in the laboratory in San Francisco ($1.13 \pm 0.15 \mu g/RBC \times 10^8$, $p < 0.01$).

(2) Lipid class distribution (Table 2) was distorted in the patients, with a decrease in the per cent of phosphatidylethanolamine of approximately one-third and an analogous increase in the per cent of phosphatidylethanolamine ($p < 0.001$) (Table 2). However, the absolute amounts of both phospholipids per cell were increased for all patients. The percentages of sphingomyelin and phosphatidylserine—inositol were not significantly altered in the patients.

(3) Analysis of the overall fatty acid composition (Table 2) showed a moderate increase in the percentage of the typical saturated fatty acid, palmitic acid (16:0), with a mean of 34.8 in the 25 patients instead of 24.2 in the controls ($p < 0.001$). There was a reciprocal reduction in the average percentage of the typical polyunsaturated fatty acid, arachidonic acid, (3.96 vs. 14.8, $p < 0.001$) in the controls. However, there were wide variations in these individual values among the thalassemic patients, and it was not possible to show correlations of MDA levels/μg of phosphorus with these fatty acid changes. As suggested by
Table 1. Hematologic Parameters and Overall Lipid Analysis of Cells From Thalassemic Patients, “Shipment Controls,” Splenectomized Controls, and Normal Controls

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Reticulocytes (%)</th>
<th>Nucleated Cells/ cu mm x 10^6</th>
<th>Days Posttransfusion</th>
<th>Approx. Total No. Transfusions</th>
<th>µg Phosphoryls/ RBC x 10^6</th>
<th>nmols MDA/ RBC x 10^6</th>
<th>nmols MDA/ g Hb</th>
<th>nmols MDA/ µg Lipid P</th>
<th>Vitamin E (mg/100 ml)</th>
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<tbody>
<tr>
<td>S.A.</td>
<td>M</td>
<td>15</td>
<td>1.3</td>
<td>83</td>
<td>9</td>
<td>120</td>
<td>1.72</td>
<td>1.61</td>
<td>560</td>
<td>0.94</td>
<td>0.22</td>
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<td>62</td>
<td>9</td>
<td>150</td>
<td>1.48</td>
<td>1.61</td>
<td>540</td>
<td>1.09</td>
<td>0.36</td>
</tr>
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<td>E.A.</td>
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<td>62</td>
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<td>50</td>
<td>1.55</td>
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<td>623</td>
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<td>0.26</td>
</tr>
<tr>
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<td>83</td>
<td>24</td>
<td>150</td>
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<td>1.96</td>
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<td>50</td>
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<td>2.77</td>
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<td>3.21</td>
<td>1578</td>
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Mean ± SD: 85 ± 42, 62 ± 34, 2.21 ± 0.50, 2.47 ± 0.68, 925 ± 302, 1.11 ± 0.13, 0.29 ± 0.18
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<td>Mean</td>
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<td>38</td>
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<td>6</td>
<td>—</td>
<td>—</td>
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<td></td>
<td>N.M.</td>
<td>F</td>
<td>17</td>
<td>—</td>
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<td>—</td>
<td>1.33</td>
<td>1.38</td>
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<tr>
<td>(6)</td>
<td>Normal controls</td>
<td>1.1 ± 0.6</td>
<td>8 ± 2.2</td>
<td>—</td>
<td>—</td>
<td>1.13 ± 0.15</td>
<td>1.22 ± 0.14</td>
<td>465 ± 51</td>
<td>1.08 ± 0.11</td>
</tr>
</tbody>
</table>

All values represent averages of two replicate determinations on each sample.

(1) Splenectomized Israeli thalassemics (asterisk indicates thalassemia intermedia).
(2) Nonsplenectomized Israeli thalassemics (asterisk indicates thalassemia intermedia).
(3) Nonsplenectomized Italian-American thalassemics.
(4) "Shipment controls." These were drawn together with the Israeli thalassemic bloods and both were processed within 48 hr of phlebotomy.
(5) Splenectomized controls. Three asymptomatic subjects with splenectomy for trauma and one patient with splenectomy for staging of Hodgkin's disease.
(6) Normal controls represent average values of blood from a group of 25 healthy random donors which were processed immediately; ± 1 SD.
<table>
<thead>
<tr>
<th>Name</th>
<th>N</th>
<th>PC</th>
<th>PE</th>
<th>SM</th>
<th>PS-PI</th>
<th>Palmitic</th>
<th>Arachidonic</th>
<th>Unsaturation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Israeli thalassemics (shipped)</td>
<td>17</td>
<td>39 (±4)</td>
<td>19 (±5.3)</td>
<td>28 (±4.2)</td>
<td>14 (±3.3)</td>
<td>3.4 (±7.5)</td>
<td>3.5 (±4.6)</td>
<td>86 (±8.1)</td>
</tr>
<tr>
<td>Normal controls</td>
<td>25</td>
<td>31 (±3.3)</td>
<td>30 (±3.7)</td>
<td>32 (±2.5)</td>
<td>16 (±4.1)</td>
<td>24.2 (±3.0)</td>
<td>14.8 (±1.2)</td>
<td>116 (±9)</td>
</tr>
</tbody>
</table>

*Palmitic acid (C16:0) and arachidonic acid (20:4) were detected as representative saturated and polyunsaturated fatty acids of the red cell membrane. The unsaturation index equals the sum of the products of the mole percentage of each fatty acid times the number of double bonds in each and represents an average indication of the extent of unsaturation in the lipids.

†Patients and controls are described in Table 1. Data represent mean values ± SD in parentheses for normal controls and Israeli thalassemics and mean ± ranges in brackets for the other groups.

PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS-PI, phosphatidylinerine–inositol.
the arachidonic acid levels (Table 2), “unsaturation indices” confirmed an over-
all decrease in the unsaturation of the thalassemic lipids.*

(4) In agreement with the results of Stocks et al., the MDA levels after perox-
ide exposure were significantly elevated in β-thalassemia major in comparison
to the controls with a mean of 924 nmoles per gram hemoglobin in the patients
versus 513 nmoles in the controls (Table 1). (Since the number of shipment con-
trols was small compared to the number of patients, the p value was calculated
according to the Mann-Whitney test.) A significant increase in MDA values
was also found when the MDA values were presented in terms of the number of
red cells (p < 0.0001). However, when calculated per microgram of lipid phos-
phorus at risk per $10^8$ cells, the mean values were not statistically different (1.11
nmoles, compared to 1.08 nmoles in the controls [Table 2]).

(5) As can be seen from Table 1, plasma vitamin E levels were decreased in
the thalassemics, and in a few cases (S.A., G.D., and L.S.) were close to zero.
The mean value was 0.27 mg/100 ml, compared to the control level of 0.65 mg/
100 ml (p < 0.001), in accord with published results. In data not shown,
plasma β-carotene levels were normal both in the β-thalassemia patients and in
the controls.

DISCUSSION

By measuring the generation under oxidative stress of increased levels of
malonyldialdehyde (MDA), a secondary breakdown product of lipid peroxides,
Stocks et al. concluded that thalassemic RBCs were more susceptible than nor-
mal cells to auto-oxidation. These conclusions were supported when an in-
crease of sphingomyelin and phosphatidylcholine and a decrease in phos-
phatidylethanolamine were found in 20 children with β-thalassemia major in
Sardinia. In an earlier study, a moderate increase in total phospholipids with
a relative decrease in the content of phosphatidic acid and phosphatidylserine
plus lysolecithin was found in a group of 29 Greek children with β-thalassemia
major. In this group, the lipid analyses were carried out at least 60 days after the
last blood transfusion.

The results of the present study show that the total membrane lipids in β-
thalassemia major, expressed as micrograms of phosphorus per cubic centimeter
of RBC, are significantly higher than control values. Within that increase in
total lipids, the percentage of phosphatidylethanolamine is reduced and phos-
phatidylcholine is increased, although the absolute amount of phosphatidyl-
ethanolamine per cell is elevated. Finally, the percentage of unsaturated fatty
acids, as exemplified by arachidonic acid and as indicated by the unsaturation
indices, was decreased in comparison to controls.

Some increase in lipid membrane may be attributed to the fact that the sam-
ples were in transit for 48 hr before being analyzed. Remotely, some plasma
lipoproteins may have become incorporated into, or onto, the membrane. How-
ever, this effect cannot be a major one, since the differences between the
normal control sent to San Francisco on two separate occasions and the fresh

*These indices were derived from total fatty acid analyses which in the interests of simplicity
are not presented here.
normal values, as well as the differences between the Israeli and American thalassemic samples, were negligible. Additionally, lipid membrane analysis of one control (E.R.) was also done on fresh blood in San Francisco and gave virtually identical results to those obtained after shipment of the same donor's blood from Jerusalem.

Another possible explanation for the high lipid values may be the presence of a large number of nucleated red cells and reticulocytes, representing a population of younger RBC. However, calculating from a nomogram obtained from determinations of RBC lipids in a series of other nonpathologic conditions with reticulocytosis*, the reticulocyte content encountered here cannot explain a total lipid increase of more than 15%. Additionally, the high lipid may be partially a function of the splenectomies which most of the patients had undergone. Cooper has shown that splenectomy may elevate normal red cell lipids by approximately 15%, and the eight nonsplenectomized patients with β-thalassemia major in the current series had total lipid phosphorus contents per RBC closer to control levels. However, our four splenectomized nonthalassemic controls showed only minimal elevations of red cell lipids, so it is unlikely that splenectomy alone can explain the observed changes. Additionally, the increased lipids may simply represent the excess and reduplicated membrane material previously observed in electron micrographs of these cells.

Severe liver disease also may produce increased red cell membrane lipids, and chronically transfused thalassemic patients often eventually develop liver disease. However, the average bilirubin level of our patients was only 2.8 with a range of 1.9 to 3.3; so it is also unlikely that liver disease severe enough to influence red cell lipids was present.

Whatever the cause of the elevated lipids, the MDA levels in most of the patients with β-thalassemia major, splenectomized or not, were much higher than controls when expressed either per gram of hemoglobin or per cell. However, when MDA was calculated per µgram of phosphorus per cubic centimeter of cells, there was no significant difference between the thalassemia patients and the controls. This finding implies that in thalassemic cells, the lipid is more susceptible to auto-oxidation, simply because there is more vulnerable lipid present in each cell in comparison to the usual peroxide defense mechanisms which are not proportionally increased. The well-known importance of the need for vitamin E in proportion to the amount of polyunsaturated lipids in the defense of biologic membranes against peroxidant threats supports this concept.

The finding of a lowered percentage of phosphatidylethanolamine, despite the increase in total amounts of all phospholipids, also supports this view, since phosphatidylethanolamine has been previously found to be the RBC membrane lipid most susceptible to peroxidation. Likewise, the reduction in arachidonic acid and the increase in palmitic acid suggests that peroxidation may have occurred. Stimulation of a membrane repair process, as previously noted in vitamin E-deficient human RBC in vitro, may have accentuated these changes in vivo.

*Shohet and Livermore, unpublished observations.
LIPID MEMBRANE PEROXIDATION

Two possibilities for inadequate peroxidant defense are present. One is that vitamin E levels in the cells or plasma are insufficient to protect the large excess of lipid membrane. Alternatively, factors such as the concentrations of glutathione peroxidase, catalase, superoxide dismutase, or even hemoglobin itself could allow intracellular peroxidant radicals to reach the membrane. In this last regard, it is of interest to note that Misra and Fridovich and Wever et al. have both obtained data that the superoxide radical is generated during the oxidation of hemoglobin. Moreover, Brunori has found that more superoxide radical is generated following oxidation of separated hemoglobin chains than with the intact molecule, implying that in thalassemia an increase in superoxide might be expected.

The first alternative initially seems attractive, since in our hands the vitamin E levels were almost uniformly decreased, and since the low vitamin E levels were probably not due to malabsorption, as shown by the normal carotene levels. However, observations by others of positive peroxide lysis tests "in spite of normal plasma vitamin E levels" in 9 of 21 children with beta-thalassemia suggest that the low plasma vitamin E levels may be due to a secondary consumption of the antioxidant consequent to the membrane oxidation, rather than a primary cause. Likewise, Stocks et al. noted that vitamin E is "by no means the only factor in antioxidant protection," since their serum alpha-tocopherol levels did not correspond with their MDA values after peroxide threat. Eventually, the difficult determination of cellular vitamin E, rather than plasma vitamin E, will be needed to assess this possibility fairly.

The second alternative is attractive in that the thalassemic cells have low MCHC values. Moreover, the greatest difference in MDA levels was found when they were expressed as MDA per gram of hemoglobin. In fact, when expressed per gram of lipid at risk, no difference between normal and thalassemic cells could be detected.* This factor could be a major reason why free radicals might find their way to the cell membrane, since the quantity of the substrate hemoglobin, which they would otherwise attack, is not adequate to act as a "sump" for these dangerous agents.

Whether this postulated intracellular protective defect is due to the reduced concentration of hemoglobin itself or to some concomitant depression of catalase, glutathione peroxidase, superoxide dismutase, or other elements of peroxide catabolism is not yet clear, although, as noted by others, the absence of significant hemolysis in acatalasia suggests that at least catalase is not crucial in protecting the cell from oxidative damage sufficient to produce hemolysis.

An additional factor which might play a significant role in lipid membrane peroxidation and decreased levels of vitamin E is related to the excess amounts of iron as a result of ineffective erythropoiesis, hemolysis, and multiple transfusions. Ferrous iron is known to be a catalyst in the nonenzymatic auto-oxidation of unsaturated fatty acids, a reaction which is specifically inhibited by alpha tocopherol. Considering the effects of iron overload as an initiator of oxidant stress, one would expect that those patients with the most marked lipid

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*This observation has been also independently noted by others (Vettore L, personal communication) and suggested as a possibility by Stocks et al.
abnormalities would have the shortest intervals between transfusions. However, it was not possible to confirm this concept in the present study, since the lipid membrane abnormalities were found to be more significant in those patients with the longest intertransfusion interval and a smaller proportion of donor blood (Table I). We have not as yet had an opportunity to study the erythrocyte lipids of nonthalassemic heavily transfused patients (e.g., aplastic anemia). When available, such data might help to clarify this point.

The present observations suggest that Heinz body formation in thalassemia does not necessarily produce membrane damage by direct, anatomically contiguous, chemical interactions. Instead, they suggest that indirect reactions, possibly mediated by free radicals and modulated by intracellular factors, may serve as an intermediate mechanism for the membrane damage in thalassemia.

REFERENCES

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