Ultrastructure of Erythrocyte Membranes in Thalassemia Major and Minor

By J. F. Hoffman, I. J. Wolman, J. Hillier and A. K. Parpart

The ultrastructure of the isolated plasma membranes (ghosts) from normal human erythrocytes has been directly studied by Hillier and Hoffman using the electron microscope. The reproducibility of the methods employed has been further evaluated and the observations extended to the membranes of erythrocytes of several other mammalian species. There thus appears adequate morphological background to apply these techniques to the study of abnormal erythrocytes and in particular to those derived from patients with thalassemia major and minor.

There are manifestly three classes of information concerning the erythrocytes found in T. major which suggest that their plasma membranes might be different from normal: the characteristically abnormal cellular shapes, the reduced in vivo survival times and the chemical composition of isolated plasma membranes. Differences in permeability could constitute a fourth class but the existing measurements (cf. Dickstein et al.) are at present only suggestive. If these gross differences from normal are expressions of alterations at the molecular level then a study of their membrane structure could reveal a morphologic basis for these differences. The evidence that these differences are, in fact, observable is presented below.

Materials and Methods

Blood from test subjects was collected from the finger tip and prepared by the method of successive hemolysis outlined by Hillier and Hoffman incorporating the modifications described by Hoffman et al. The ghosts were fixed in 0.1 per cent phosphotungstic acid for three minutes, washed in distilled water, dried in air, and shadowed with chromium.

The preparations were examined using an RCA-EMU electron microscope fitted with an aperture-free double objective lens. All micrographs appearing in this paper (except those presented in Plate I) were taken at an electron optical magnification of 15,400X and enlarged photographically to a final magnification of 75,000X. The micrographs in Plate I were taken in the lower magnification range of the double objective without, however, re-adjustment of the compensation for use in this range.

From the Biology Department, Princeton University; The Children's Hospital of Philadelphia; Department of Pediatrics, School of Medicine, University of Pennsylvania; and RCA Laboratories Division, Princeton, New Jersey.

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Plate I

The figures on Plate I are micrographs of typical fields of two different ghost specimens prepared by the method of successive hemolysis. The distribution of size of the ghosts in figure 1 is quite narrow; in figure 2, considerable variation is apparent. The ghosts displaying many gross particles are probably reticulocytes. Magnification of both figures is 3000X. Shadowed.

Fig. 1.—T. minor. Ghosts prepared from the blood of patient M.B. (7304a)
Fig. 2.—T. major. Ghosts prepared from the blood of patient O.B. (7284a)
PLATE I
(See legend, facing page)
TABLE 1.—Summary of Pertinent Clinical Data on the Thalassemia Patients and Normal Control at Time Electron Microscopy Was Done

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs.)</th>
<th>Sex</th>
<th>Clinical severity*</th>
<th>No. of days since last transfusion</th>
<th>Hgb., gm. per 100 ml.</th>
<th>RBC, c.mm.</th>
<th>Retic., %</th>
<th>Prior splenectomy</th>
<th>Morphologic changes in red cells†</th>
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<tbody>
<tr>
<td>Thalassemia major</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>O. A.</td>
<td>11</td>
<td>F</td>
<td>++++</td>
<td>24</td>
<td>46</td>
<td>4.5</td>
<td>2.17</td>
<td>4.2</td>
<td>+</td>
</tr>
<tr>
<td>O. B.</td>
<td>4</td>
<td>M</td>
<td>+++</td>
<td>23</td>
<td>45</td>
<td>6.9</td>
<td>2.72</td>
<td>4.2</td>
<td>+</td>
</tr>
<tr>
<td>O. T.</td>
<td>8</td>
<td>M</td>
<td>++++</td>
<td>48</td>
<td>31</td>
<td>6.6</td>
<td>2.62</td>
<td>5.0</td>
<td>+</td>
</tr>
<tr>
<td>O. S.</td>
<td>1</td>
<td>M</td>
<td>+++</td>
<td>15</td>
<td>11</td>
<td>6.5</td>
<td>2.86</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>O. C.</td>
<td>2</td>
<td>M</td>
<td>+++</td>
<td>23</td>
<td>4</td>
<td>7.7</td>
<td>3.12</td>
<td>2.8</td>
<td>0</td>
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<tr>
<td>Thalassemia minor</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>M. A.</td>
<td></td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>11.6</td>
<td>5.06</td>
<td>1.3</td>
<td>0</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>—</td>
<td>11.0</td>
<td>5.02</td>
<td>1.5</td>
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<tr>
<td>M. T.</td>
<td></td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>11.5</td>
<td>5.45</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>M. S.</td>
<td></td>
<td>F</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>11.6</td>
<td>5.56</td>
<td>0.5</td>
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<tr>
<td>F. S.</td>
<td></td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>13.9</td>
<td>6.72</td>
<td>0.95</td>
<td>0</td>
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<tr>
<td>F. C.</td>
<td></td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>14.0</td>
<td>5.88</td>
<td>0.7</td>
<td>0</td>
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<tr>
<td>M. C.</td>
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<td>F</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>10.2</td>
<td>4.48</td>
<td>0.7</td>
<td>0</td>
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<tr>
<td>Control</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>JFH</td>
<td></td>
<td>M</td>
<td>0</td>
<td>0</td>
<td>—</td>
<td>14.9</td>
<td>5.05</td>
<td>1.5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Pallor, splenomegaly, characteristic roentgenographic bone changes, typical facies, persistent anemia requiring repeated transfusions. Both parents of all children were of Italian ancestry. The parents not studied by electron microscopy also had the changes in the circulating red cells characteristic of Thalassemia minor.

† Target cells, oval cells, achromia, anisocytosis, poikilocytosis, stippling. Circulating nucleated red cells in thalassemia major subjects only.

At the same time that blood was removed for study by electron microscopy, samples were also obtained from the patient for routine hematologic examination in the laboratory of one of the authors.* Table 1 presents the summary of these findings; in addition some clinical observations are also given. For each patient the basis for diagnosis was assessed and followed for a number of years prior to the present study.

Five families were chosen for this study in which at least one child is known to have Thalassemia major and both parents, Thalassemia minor. For reference to patients where

* I. J. Wolman at the Children's Hospital of Philadelphia.

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PLATE II

Fig. 3.—T. minor. Representative ghost from the blood of patient M.C. Compare the surface texture of this ghost with that of Fig. 4. Magnification 75,000X. Shadowed. (7573c)

Fig. 4.—T. major. Representative ghost from the blood of patient O.C. The surface texture of this ghost is distinctly different from the ghost presented in figure 3. Typical also is the presence of many holes apparent in the surface texture. This is true of all of the ghosts prepared from T. major bloods (see figs. 5, 6, and 7). The frequency of the appearance of holes in the surface texture of ghosts derived from T. minor bloods (figs. 3, 8, 9, 10, 11 and 12) is greatly reduced in comparison. The formation of holes is ascribable to a difference in the reaction of the two types of ghosts to the surface forces developed during air drying. High density particles (HDP) are evident within the ghost area. HDP can also be seen in figures 5, 6 and 7. Magnification 75,000X. Shadowed. (7588c)
PLATE II
(See legend, facing page)
the diagnosis is T. major the letter O is used; for T. minor, M refers to the mother and F to the father. The last letter denotes the family. A total of five children, five mothers and two fathers have been studied.

Results

Plate I shows two electron micrographs at the same magnification of typical fields of ghosts isolated by the procedures referred to above. Figure 1 represents ghosts derived from a patient with T. minor (M.B.) and figure 2 with T. major (O.B.). The distribution of size of the ghosts in figure 1 is quite narrow while considerable variation is apparent in figure 2. This means that the distribution of red cell size observed in whole blood smears by standard microscopic procedures is maintained in the preparation of their ghosts. This was confirmed in a separate higher resolution study in which the surface area of normal human ghosts, comparable to but less crowded than those in figure 1, was determined with the aid of a polar planimeter. It was found that the surface area of these ghosts was not significantly different from the surface area of the intact cells from which they were derived.

The ghosts of Plate I containing gross, relatively large particles are probably reticulocytes according to the correlation studies of Hoffman. He observed that the surface texture of the ghosts of reticulocytes of normal subjects are indistinguishable from the ghosts of their mature erythrocytes. This finding has also been true of all of the T. minor bloods examined but not in T. major bloods. The T. major patients used in this study (see table I) were receiving periodic transfusions of normal blood. The resulting preparation of ghosts thus contained a mixed population of host and donor ghosts. It was found that the ghosts from this mixture were heterogeneous with respect to their surface textures but could be placed into two distinct groups. One group proved to be indistinguishable from normal ghosts (and also the ghosts of T. minor) both in surface texture and in the uniformity of size distribution. The remaining group was correlated closely with the ghosts of variable size which contained the gross particles. In addition, the former group of ghosts did not exhibit high density particles (HDP) (cf. Hoffman et al.) but the latter group did. The assumption was therefore made that the ghosts containing the gross particles, characterized on the basis of their surface textures, were reticulocytes and belonged to the host. These selected ghosts are the only ones which concern us here.

The surface texture of T. minor ghosts are seen in Plates II and IV, figures 3, 8, 9, 10, 11 and 12 at a magnification of 75,000X. Plates II and III, figures 4, 5, 6 and 7 illustrate the surface texture of T. major ghosts also at 75,000X. For comparison figures 13 and 14 (again at 75,000X) are included which are ghosts from normal human blood (JFH). All of the electron micrographs shown in the plates are "representative" in the restricted sense defined by Hoffman.

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PLATE III

Fig. 5.—T. major. Representative ghost isolated from patient O.A. Magnification 75,000X. Shadowed. (7315c)

Fig. 6.—T. major. Representative ghost isolated from patient O.S. Magnification 75,000X. Shadowed. (7473d)

Fig. 7.—T. major. Representative ghost isolated from patient O.T. See text for discussion of the differences between the surface texture of this ghost and those illustrated in figures 4, 5, and 6. Magnification 75,000X. Shadowed. (7257b)
PLATE III
(See legend, facing page)
Upon examination it is seen that within each family the ghosts of the parents' blood (T. minor) are distinctly different from the ghosts of the child's blood (T. major). Further, the comparison of all the illustrated T. major ghosts shows that they are quite similar to each other (with the exception of case O.T.). This also holds when all of the T. minor ghosts are likewise compared. Thus, the surface textures of the ghosts of T. majors appear to be different from those from T. minors.

DISCUSSION

The primary problem is to decide if the differences noted above between T. minor and T. major are, in fact, real. To answer this question adequately it is necessary to eliminate certain variables in addition to evaluating the variation presumably introduced by the preparative technic itself.

One possibility for these differences, for instance, could be correlated with the age of the individuals studied. However, electron microscopic examination of ghosts from the blood of normal children and children with nutritional anemias has not revealed any readily observable differences from normal adults. In addition, the ghosts derived from T. major represent the most extreme differences that have been observed even when compared to other hereditary hemolytic anemias in children and in adults. These same findings obtained when the sex of the patient is considered; that is, no differences in the surface textures of ghosts referable to the sex of the patient have been observed.

Upon comparing figure 3 and all the figures on Plate IV with those on Plate V the amount of variation that is apparent lies within the variability referable to the preparative technique itself (Hoffman⁶). This is further emphasized if the comparison is extended to include Plate VIII of Hoffman.⁶ For these and other reasons discussed by Hoffman,⁶ normal human blood (JFH) was always prepared at the same time and under as identical conditions as possible with the Thalassemia bloods and consequently served as an internal control. Figure 13 is a ghost from the control blood prepared at the same time as those from cases M. B., O. B., M. T., O. T., M. A. and O. A. In like manner figure 14 is from the control blood prepared at the same time as cases M. S., F. S. and O. S. From a study of these representative micrographs the inference is drawn that the surface texture of ghosts derived from T. minor patients is not distinguishable from the surface texture of ghosts from normal blood within the limits of the technics employed.

The reality of the differences observable between T. major and T. minor can be deduced in the following manner. When the individual bloods of a particular family are prepared at the same time differences between the ghosts of T. major and T. minor red cells are easily discernible. All of the representative ghosts of T. minors appear similar to each other and to the control; all of the selected ghosts derived from T. major (with the exception of those of O. T.) appear similar to each other but are distinctively different from those of T. minor and the control.

PLATE IV

Figs. 8, 9, 10, 11, and 12.—T. minor. Representative ghosts derived from the blood of patients F.C., M.A., M.S., F.S., and M.T., respectively. Note the similarity in the appearance of the surface texture of these ghosts. Magnification of all figures is 75,000X. Shadowed. (7583d, 7388d, 7479d, 7497d, 7248e)
PLATE IV
(See legend, facing page)
These differences between T. minor and T. major were observed consistently whether the specimens for comparison were prepared at the same time or on different days. Thus, it may be concluded that the surface ultrastructure of T. major ghosts is different from T. minor ghosts.

It is of interest to consider the T. major patient O. T. The fact that the surface texture of this patient's ghosts (fig. 7) are recognizably different from the other T. majors studied as well as the T. minors and controls does not necessarily weaken the conclusions reached above. The appearance of the surface texture suggests that these ghosts are intermediate between T. minor and T. major and places the difference as representing biological variation (see below).

Sundharagati and Wright present the only reported instance of an electron microscopic investigation of T. major. Their low magnification study of ghosts prepared somewhat differently make comparison to our results difficult as pointed out by Hillier and Hoffman. It is pertinent, in view of their results, however, to consider fold formation observable in the air-dried specimens. We have found no correlation of the type of folding with any particular blood or treatment. The pattern of folds (star-shaped, circular-ring or no folds) could be seen on different portions of the same specimen. It appeared that the type of folding obtained was dependent upon the manner in which water was removed from the specimen.

In terms of the plaque theory presented by Hillier and Hoffman and Hoffmans the interpretation of the differences between T. major and T. minor appears to consist essentially of alterations in both plaque size and arrangement with little contribution of a change in plaque shape. Viewed in this fashion and taking into account the increased lipid content found by Erickson et al., the plasma membrane in T. major could represent these molecules in an assemblage different from normal.

Thalassemia minor and T. major appear to represent heterozygous and homozygous conditions, respectively. Thalassemia, considered as multiple effects of a single gene, could, for the erythrocyte of T. major, be represented by the following alterations: (1) The distinctive ultrastructure of the plasma membrane (this would mean that the less extreme case of O. T. (fig. 7) is caused by a more limited expression of the thalassemia gene). (2) The presence of high density particles (the HDP of Hoffman et al.). (3) The presence of gross particles probably identifiable as the “inclusion bodies” of Minnich et al. (4) The presence of an alkali resistant hemoglobin and (5) The characteristics summarized in the introduction. The new properties of the erythrocyte of T. major described in this paper together with the other available information suggest that an abnormally constructed red cell plasma membrane is in part responsible for the Thalassemia major syndrome.

**Summary**

The plasma membranes of erythrocytes derived from Thalassemia major (five cases) and minor (seven cases) were isolated and studied by electron microscopy.

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**PLATE V**

Figs. 13 and 14.—Normal control. Representative ghosts isolated from the blood of one normal human subject on different days. See text for discussion. Magnification 75,000X. Shadowed. (7399a, 907d)
PLATE V
(See legend, facing page)
MEMBRANE STRUCTURE IN THALASSEMIA

It was found that the surface texture of ghosts from all T. minor bloods appeared similar and indistinguishable from ghosts of normal human blood. The surface texture of ghosts from all T. major bloods likewise were quite similar but distinctly different from T. minor and normal ghosts. These differences were observable whether the specimens for comparison were prepared at the same time or on different days. The results indicate that the morphological characteristics observed in T. major ghosts constitute an expression of an alteration in the molecular structure of their plasma membranes.

SUMMARIO IN INTERLINGUA

Le membranas del plasma de erythrocytos obtenite ab (cinque casos de) Thalassemia major e (septe casos de) Thalassemia minor esseva isolate e studiate per microscopia electronic. Esseva constatate que le textura superficial de spectros ab omnie sanguines de T. minor esseva simile e non distinguibile ab spectros ab sanguine human normal. De mesmo, le textura superficial de spectros ab omne sanguines de T. major esseva similissime sed distinctemente differente ab spectros a T. minor e ab spectros normal. Iste differentias esseva observabile sin reguardo a si le specimens pro le comparation esseva preparate al mesme tempore o in dies differente. Le resultatos imsdica que le characteristicas morfologic observe in spectros a T. major constitue le expression de un alterations in le structura molecular de lor membranas plasmatic.

REFERENCES

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