A CLINICAL AND EXPERIMENTAL STUDY OF THE ERYTHROCYTE ULTRASTRUCTURE MEMBRANE WITH THE ELECTRON MICROSCOPE¹

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Increasing interest is being focused on the various modifications and environmental influences which may render red blood cells progeric and susceptible to one of the destructive mechanisms. In addition to the study of the intact erythrocyte and its environment, investigations of the two primary components, *i.e.*, the hemoglobin and the ultrastructure, have been particularly fruitful in the last fifteen years. The surface ultrastructure has been fairly well defined chemically and physically (1). The more recent work on identification of genetically different chemical types of human hemoglobin has thrown new light on the hemoglobin-ultrastructure relationship, but has also revealed additional problems (2-4).

Most of the previous observation on erythrocyte ultrastructure by means of the electron microscope have been confined to a single type of red blood cell (5-8). It was our intention to make a broader study of the ultrastructure with the hope of obtaining a better understanding of erythrocyte destruction. The following types of red blood cells were included: Normal erythrocytes and erythrocytes from patients with various hematologic diseases; erythrocytes modified by sensitization, enzyme treatment (trypsin), roentgen radiation, *in vitro* and *in vivo*, storage, incubation, treatment with various chemicals, and varying pH.

MATERIALS AND METHODS

I. Preparation

A. General. Unless otherwise specifically noted, the following procedures were used:

1. *Collection and washing of erythrocytes:* Four ml. of intravenous blood were collected in 1 ml. ACD solution

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(Baxter). After removal of plasma by centrifugation, blood cells were washed three times with saline.²

2. *Preparation of membranes:* Freshly collected, washed erythrocytes were hemolyzed by addition of 100 to 200 volumes of distilled water (pH 6.5). The suspension was allowed to stand at room temperature for 10 minutes. Membranes were separated by centrifugation and washed six times with distilled water.

3. *Preparation of screens for electron microscope:* One drop of a thin suspension of the membranes or erythrocytes was placed on a Formvar³ membrane screen and dried at room temperature. *Fixatives were not used.*

B. Preparation of membranes from one particular case of idiopathic acquired hemolytic anemia (W. T.).

Sixty per cent of the red cell population in this case (W. T.) consisted of spherocytes. In order to obtain a higher yield of spherocytic membranes, the freshly collected sample was allowed to stand for 10 minutes at room temperature. Spontaneous hemolysis of a large portion of the red cells occurred. The hemolyzed cells were assumed to have been spherocytes. The remaining intact erythrocytes were then removed by slow centrifugation (1,000 rpm. for 5 minutes). The major portion of the membranes from hemolyzed erythrocytes were thus retained in the supernatant plasma. Resuspension in saline of the intact erythrocytes, followed by further slow centrifugation, yielded additional spherocytic membranes. The membranes were then washed six times with distilled water.

C. Modified erythrocytes and membranes.

1. *Chemical treatment:* The following chemicals were used in treating erythrocytes and membranes: osmic acid 1 per cent, formalin 1 per cent and 10 per cent; 1 per cent solutions of copper sulfate, lead nitrate, ferric sulfate, mercuric chloride and silver nitrate.

- (a) Two-tenths ml. of normal packed washed RBC were incubated with 5 ml. of the chemical solution for 10 minutes at room temperature.
- (b) Five ml. of normal membrane suspension were incubated with 5 ml. of chemical solution at room temperature. Membranes were then washed three times with distilled water.

² All saline is physiologic in this study.

³ A resin manufactured by the Shawinigan Products Corp., New York, N. Y.

2. *Effect of pH*: Normal, washed erythrocytes were hemolyzed as in section A above, except that altered pH values of the distilled water, as checked with a Beckman pH meter, were used.

3. Trypsin treatment:

(a) Treatment of the intact erythrocyte: One volume of normal, washed red cells was incubated with one and one-half volumes of trypsin⁴ solution at 37° C. for 30 minutes, then washed three times with saline. Concentrations of trypsin ranged from 0.1 to 4.0 per cent.

(b) Treatment of normal membranes: One drop of the desired trypsin solution was placed on the erythrocyte membrane previously dried on the Formvar screen. Following incubation at 37° C. for half an hour, the trypsin solution was decanted off, and the screen allowed to dry at room temperature.

4. *Sensitization of erythrocytes*: Group A and B human erythrocytes were sensitized with anti A and anti B sera (Lederle) in various dilutions. Two-tenths ml. of washed, packed cells and 1 ml. of the serum dilution were incubated for 30 minutes at 37° C. Following sensitization, the cells were washed three times, and the membranes prepared as outlined under section A above.

5. Irradiation of erythrocytes:

(a) *In vivo*. Domestic rabbits were given 850 to 900 r total body irradiation. Blood specimens were taken by cardiac puncture at 24 hours postirradiation, and every 48 hours thereafter as long as the animal survived.

(b) *In vitro*. Normal erythrocytes were washed five times with saline. One ml. of a 1:1 cell-saline suspension was placed in a paraffin coated tube (1 cm. diameter) and irradiated in doses ranging from 24,000 r to 96,000 r (140 KV, no filter, 8 cm., 7 mA, 2087 r per min.). After irradiation the erythrocytes were hemolyzed immediately, and the membranes prepared in the usual manner.

6. *Stored erythrocytes*: Bank bloods, collected in ACD solution, and stored at 4° C. were examined weekly for as long as 20 weeks.

7. *Influence of temperature on normal erythrocytes and membranes*: The erythrocytes or membranes were treated in the following manner:

- (a) Sterile, defibrinated blood samples were incubated at 37° C. for ½ to 24 hours.
- (b) Saline suspensions of normal, washed erythrocytes were heated for 30 minutes at 56° C.
- (c) Normal, washed red cells were hemolyzed in distilled water at 56° C. for 10 minutes.

(d) Normal membranes suspended in distilled water were heated at 56° C. for 30 minutes.

(e) Normal membranes were stored in distilled water (pH 6.5) at 4° C. for 21 days.

II. Observations

Fifty to one hundred membranes or red cells were examined in each specimen. In addition to the fluorescent screen study, the observations reported here were also based on the detailed study of approximately 2,400 electron microphotographs.

Observations and photographs were made with the electron microscope type EMU, RCA. In the instances in which chromium and gold shadow casting were used, the technic was that described by Wyckoff (9). Details are given in the captions.

RESULTS

I. Normal erythrocyte membranes

Forty-three samples of erythrocytes from thirty healthy individuals were examined. Only minimal differences in the membranes of the red cell spectrum could be detected; thus, for example, it was not possible to distinguish the young from the aged circulating cell.

The normal erythrocyte membrane appeared as a thin-walled double disc, comparable to the flattening of a round balloon. The diameter was slightly larger than that of the intact red cell. The surface presented a finely granular appearance (Figure 1).⁵ The great majority of the membranes showed no holes; however, rarely one (Figure 2) or more holes (Figure 3) were evident. Three of the 43 specimens showed fold formation in 1 to 3 per cent of the membrane population. Fold formation is discussed more fully in a following section. Unhemolyzed erythrocytes often showed a less dense center in electron micrographs, evidence of less hemoglobin in this area. In partially hemolyzed erythrocytes, a density of hemoglobin was seen at the periphery (Figure 4).

⁵ Recent reports by Bessis, Ponder and their associates (20, 21, 22) that have come to our attention since the preparation of this manuscript have described a most interesting structure that they observed in erythrocyte ultrastructure membranes with the electron micrograph technics. This filamentous structure apparently originates in small craters of the ultrastructure and when observed on the periphery gives a whiskered appearance. They called these hematexodies. These were not observed in our preparations; this was probably due to our more intense washing of the membranes.

⁴ Difco trypsin, 1:250 activity.

PLATE I

- FIG. 1. NORMAL ERYTHROCYTE MEMBRANE (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)
FIG. 2. NORMAL ERYTHROCYTE MEMBRANE (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)
FIG. 3. NORMAL ERYTHROCYTE MEMBRANE (WITHOUT SHADOWING)
FIG. 4. PARTIAL HEMOLYZED ERYTHROCYTE WITH RESIDUAL HEMOGLOBIN IN THE PERIPHERY (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

PLATE II

II. *Erythrocyte membranes from cases of hemolytic syndromes, leukemia, polycythemia vera, malignant diseases, tuberculosis, and iron deficiency anemia*

A. *Hemolytic anemia*

1. *Acquired hemolytic anemia.* Twenty-three specimens from 11 cases of acquired hemolytic anemia were studied. In every case, incomplete antibodies were demonstrated by the anti-human globulin (Coombs) test and/or by the trypsinized red cell test. Changes were observed in 10 to 50 per cent of the erythrocyte population in 9 of the 23 specimens. These changes consisted of varying degrees of thickening of the membrane accompanied by irregular hole formation. Hemoglobin fragments were frequently attached to the surface (Figures 5, 6 and 7). When multiple examinations of membranes from a single case of acquired hemolytic anemia were repeated over a period of months, the same changes were constantly observed.

2. All of the five cases of spherocytosis had undergone splenectomy several years previously. Separation and recognition of spherocytic membranes were less effective than in the cases of acquired hemolytic anemia.

3. The two specimens from the single case of thalassemia major showed the changes which were qualitatively and quantitatively similar to those in acquired hemolytic anemias (Figure 8).

4. Four specimens from two cases of mild sickle cell anemia showed no abnormalities.

B. *Leukemia*

In only 9 of 55 cases of chronic leukemia were modifications in the erythrocyte membranes evident. In eight of these cases, 10 to 30 per cent of the membranes showed changes, while in a case

associated with frank intermittent hemolytic episodes, 90 per cent of the membranes appeared abnormal. Large holes with a diameter equal to one-third of that of the membrane were frequently noted (Figure 9). The impression gained was that many of these ultrastructures had marked weakness of the wall and a high susceptibility to even slight trauma. In 11 of the 55 cases, fold formations were noted in 5 to 15 per cent of the total membranes.

C. *Polycythemia vera*

Five specimens from four cases were examined. In two of these the membranes showed numerous small, irregular holes in less granular than normal stroma (Figure 10). This particular pattern was not seen in any of the other clinical or experimental material.

D. *Malignant diseases and tuberculosis*

No abnormal changes were noted in the membranes from 24 cases of these diseases.

E. *Iron deficiency anemia*

A thin fold formation was evident in 10 per cent, 50 per cent, and 90 per cent of the erythrocyte membranes in three cases of iron deficiency anemia (Figure 11). In the case with 90 per cent fold formation, five weeks of iron therapy effected an elevation in both the erythrocyte and hemoglobin levels with a diminution in the incidence of fold formation to 70 per cent. Unfortunately, further follow-up on this patient was not possible.

III. *Modified erythrocytes*

A. *The fold formation of the membrane* as first observed in the iron deficiency anemias, stimulated our interest. Previously, several investigators

PLATE II

FIG. 5. ERYTHROCYTE MEMBRANE OF THE SPHEROCYTE FROM ACQUIRED HEMOLYTIC ANEMIA (WITHOUT SHADOWING)

FIG. 6. MEMBRANE OF THE SPHEROCYTE FROM THE SAME SAMPLE OF FIG. 5 (CHROMIUM SHADOWING, 31 MG. 12 INCHES, ANGLE 18°)

FIG. 7. MEMBRANE OF THE SPHEROCYTE (HIGHER MAGNIFICATION, CHROMIUM SHADOWING, 31 MG. 12 INCHES, ANGLE 18°)

FIG. 8. MEMBRANE FROM A CASE OF THALASSEMIA MAJOR (WITHOUT SHADOWING)

FIG. 9. MEMBRANE FROM A CASE OF CHRONIC MYELOID LEUKEMIA (WITHOUT SHADOWING)

FIG. 10. MEMBRANE FROM A CASE OF POLYCYTHEMIA VERA (WITHOUT SHADOWING)

PLATE III

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noted fold formation in all of the specimens they examined; however, they reported using osmic acid or formalin as fixatives (12-14). Since fixatives denature the surface protein, the elasticity of the membrane may be destroyed. It seemed pertinent to the problem to study the effects of these agents as well as those of marked lowering of the pH and other fixative agents which produce similar denaturing effects.

Osmic acid 1 per cent—All the membranes treated showed fold formation. When normal intact erythrocytes were treated, there was no hemolysis; however, there was marked contraction of the entire cell to approximately half the normal diameter (Figure 12). Some cells similarly treated showed crenation and agglutination (Figure 13). Folds were not present in the wet preparations of erythrocyte membranes when observed under the light microscope, but were evident after drying (Figure 14). In another experiment, membranes on a wet screen were exposed to osmic acid vapor and showed fold formation after drying. Membranes dried previous to treatment with the vapor showed no folding. These observations suggest that the folds developed during the drying process.

Formalin 1 per cent and 10 per cent—Membranes treated with formalin showed folding and coarse granulation suggestive of a coagulation effect of the surface structure (Figure 15).

Salts of heavy metals—All membranes treated with copper sulfate, lead nitrate, ferric sulfate, mercuric chloride and silver nitrate showed varying degrees of folding (Figure 16). Intact erythrocytes treated with mercuric chloride and silver nitrate showed no hemolysis; there was contraction with some shifting of the internal hemoglobin and "cracking" of the intact cells (Figure 17). Fer-

ric chloride treatment produced a swirling type of fold (Figure 18).

Hemolysis of normal erythrocytes in distilled water of various pH—Marked fold formation in the membranes resulted from treatment with distilled water at pH 3.0 (Figure 19). At pH 1.5, folds were delicate and numerous (Figure 20). At pH 10.4, there was marked stomatolysis with stringlike formation (Figure 21). Hemolysis of a sample at pH 11.0, and subsequent lowering of the pH to 7.0, produced fragmentation of the membrane, with interspersed masses of hemoglobin (Figure 22). Normal membranes were subjected to distilled water with a pH of 1.5 for 10 minutes. The membranes showed disintegration similar to, but not as severe as, that obtained at pH 11.0 and a subsequent correction to a pH of 7.0.

IV. Trypsinized erythrocytes

Trypsinized erythrocytes (1) have proved to be a useful tool in demonstration of incomplete antibodies (15); (2) are more easily phagocytized than normal erythrocytes (16); and (3) have a shortened life span in the experimental laboratory animal (17). These characteristics led to their inclusion in this study.

Twenty-eight specimens of trypsinized red cells were examined. Two methods of trypsinization were employed: 1) treatment of the cells in suspension and 2) treatment after the membranes had been "filmed" on the Formvar screen. Various concentrations from 0.1 per cent to 4 per cent trypsin were used.

Membranes prepared from red blood cells treated by the first method showed no change from normal when the lower concentrations of trypsin were used. Only when cells were tryp-

PLATE III

FIG. 11. ERYTHROCYTE MEMBRANE FROM A CASE OF IRON DEFICIENCY ANEMIA (WITHOUT SHADOWING)

FIGS. 12-13. NORMAL ERYTHROCYTES TREATED WITH 1.0 PER CENT OSMIC ACID, 10 MIN. (WITHOUT SHADOWING)

FIG. 14. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH 1.0 PER CENT OSMIC ACID, 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 15. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH 10.0 PER CENT FORMALIN 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 16. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH 1.0 PER CENT COPPER SULPHATE SOLUTION, 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

sitized with 4 per cent trypsin solution did the membranes show modifications. Erythrocyte membranes from several cases of chronic leukemia which showed no evidence of clinical hemolysis were much more susceptible to trypsin treatment than were normal cells (Figure 23). Two per cent trypsin solution was used in these comparative studies.

Trypsin produced marked changes in the membranes even in a low concentration (0.1 per cent) if the membranes were dried before treatment. The changes were in proportion to the concentrations of trypsin (Figure 24).

V. Sensitized erythrocytes

Sensitized erythrocytes showed crenation and agglutination under the light microscope. In the 35 specimens studied, various abnormalities were manifested (Figure 25). Following weak sensitization the membranes appeared thinner than normal and showed some folding. Strong sensitization increased coarseness and granular changes.

VI. Irradiated erythrocytes

A. Thirty specimens from rabbits which had received 850 to 900r total body irradiation showed no change from the normal.

B. Thirty-two specimens of *in vitro* irradiated erythrocytes were studied. In only one, irradiated with 48,000 r, were variations consisting of coarse granulation and cracks noted (Figure 26). These findings are at variance with those of Zacek and Rosenberg (18) who noted marked ultrastructure modifications. They hypothesized that the irradiation caused changes in the lipoprotein. Halberstaedter and Goldhaber (19) demonstrated

changes with irradiation as noted with chemical studies; they also claimed that changes were dependent on the concentration of erythrocytes and the character of the suspension medium. Our technic was identical with that of Zacek and Rosenberg with the exception that physiologic saline was substituted for Ringer's solution. The Halberstaedter and Goldhaber concentrations were less than the 1:1 suspensions used here.

VII. Stored erythrocytes

Stored erythrocytes were studied in 43 specimens. No correlation between the changes and the duration of storage was demonstrated. Cells stored for 120 days did not show significant modifications. The few membranes that did show changes demonstrated a thinner periphery. This observation is similar to previous leptoscopic study of normal cells which showed that the ultrastructure in the region of the biconcavities was thicker than that of the periphery (1) (Figure 27).

VIII. Influence of temperature on normal erythrocyte membranes

A. Red cells incubated under sterile conditions in both plasma and defibrinated blood for varying periods from $\frac{1}{2}$ to 24 hours at 37° C. showed no changes in their membranes. It was thought that minimal differences might be detected inasmuch as 24 hours incubation did produce differences in the osmotic fragilities of erythrocytes from acquired hemolytic anemia and hereditary spherocytosis.

B. Erythrocytes in saline heated for 30 minutes at 56° C. showed "coagulation" and disintegration of their membranes with hemoglobin attached (Figure 28).

PLATE IV

FIG. 17. NORMAL ERYTHROCYTE TREATED WITH 1.0 PER CENT MERCURIC CHLORIDE SOLUTION, 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 18. NORMAL ERYTHROCYTE MEMBRANE TREATED WITH FERRIC CHLORIDE 0.01 M SOLUTION FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 19. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 3.0 FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 20. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 1.5 FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 21. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 10.4 FOR 10 MIN. (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 22. MEMBRANE FROM NORMAL ERYTHROCYTE HEMOLYZED IN DISTILLED WATER pH 11 FOR 10 MIN., THEN THE pH WAS CORRECTED TO 7 (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

PLATE V

C. Erythrocytes hemolyzed in distilled water at 56° C. for 10 minutes also demonstrated the "coagulation" phenomena and disintegration.

D. Membranes suspended in distilled water and heated at 56° C. for 30 minutes showed changes similar to those observed in the two above conditions.

E. Storage of normal membranes in distilled water (pH 6.5) at 4° C. for 21 days occasioned no change from the normal.

COMMENTS

In assessing the importance of the observations reported here, care must be taken to avoid absolute correlations and conclusions based on phenomena which may be essentially of an artifactual nature. Even if they are artifactual, it is quite possible that they may reflect some other acquired or inherited phenomena. The quantitative differences that were seen in much of the material from pathologic sources when compared with the control material from normal healthy sources do allow for a certain degree of hypothesizing. The following hypothesis as to the folding mechanism is offered: During the process of hemolysis, osmosis causes some distention of the red cell. Normally, the ultrastructure is resilient, and there is contraction before drying is complete. If the elasticity is destroyed, (as by fixatives, sensitization, trypsinization or treatment with salts of the heavy metals) or is inherently diminished (as may be in iron deficiency anemia) there is failure of contraction and the membranes fold together on the surface with the folds becoming visible. The high percentage of folding in some of the clinical material (which received identical preparation as the normal controls) could influence one to project the

possibility that fold formation could be due to a defective ultrastructure architecture.

Similarly, the significance of holes in the membranes is uncertain. Although they, as in the case of folds, are present in a small percentage of normal erythrocytes, their marked occurrence in several cases of acquired hemolytic anemia, thalassemia major, and chronic leukemia suggests a possible role in the hemolytic process. The question arises: Is hemoglobin expelled through holes? Sasagawa, Hosomi, and Mani (10), after observing the process of hemolysis with the phase contrast microscope, concluded that hemoglobin may be liberated by several means: (1) a large crack in the membrane (as seen in Figure 11); or (2) by bursting out of one or a few "spots" on the membrane (as in Figures 5, 6 and 7); or (3) by transudation out of the whole surface of the cell through numerous "pores" (as in Figure 10). It is evident, however, that further studies are necessary before such correlations can be soundly made.

SUMMARY

The erythrocyte ultrastructure membranes from normal individuals, individuals suffering from various hematologic diseases and from modified erythrocytes were studied with the electron microscope. The following observations were made:

1. Normal erythrocyte membranes showed a finely granular surface. Only rarely were holes or fold formations (1 to 3 per cent) evident.

2. Many of the red cell ultrastructures from cases of acquired hemolytic anemia, hereditary spherocytosis, thalassemia major, chronic leukemia, and polycythemia vera showed varying modifications such as increased coarseness and hole and fold formation.

PLATE V

FIG. 23. MEMBRANE OF NORMAL ERYTHROCYTE MODIFIED BY 4.0 PER CENT TRYPSIN (CHROMIUM SHADOWING, 31 MG. 12 INCHES, ANGLE 18°)

FIG. 24. DRY NORMAL ERYTHROCYTE MEMBRANE DIGESTED BY 0.5 PER CENT TRYPSIN (WITHOUT SHADOWING)

FIG. 25. MEMBRANE OF NORMAL ERYTHROCYTE MODIFIED BY SENSITIZATION (WITHOUT SHADOWING)

FIG. 26. ERYTHROCYTE MEMBRANE AFTER IRRADIATION 48,000 r (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

FIG. 27. MEMBRANE FROM STORED ERYTHROCYTE (SEVEN DAYS) (WITHOUT SHADOWING)

FIG. 28. MEMBRANE FROM HEATED ERYTHROCYTE IN NORMAL SALINE 56° C. FOR 30 MIN. BEFORE HEMOLYSIS (GOLD SHADOWING, 10 MG. 12 INCHES, ANGLE 11°)

3. The fold formations that were seen in many membranes from patients with iron deficiency anemia, and chronic leukemia led to an experimental study of the production of fold formation. Experimentally, fixatives such as formalin and osmic acid, salts of heavy metals, lowering of the pH below 3.0, intense sensitization and trypsinization also produced folding of the ultrastructure. Similar treatment also produced varying degrees of coarseness, cracking, holes, and disintegration.

4. Irradiated erythrocytes *in vivo* showed no change. In only one of thirty-two specimens irradiated *in vitro* did the membranes show modifications. Stored erythrocytes and erythrocytes incubated at 37° C. for 24 hours also showed no change.

5. The significance and interpretation of the modifications observed in both the clinical and experimental material must be guarded pending further study.

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