Morphology of Normal Erythrocyte and Acanthocyte Using Nomarski Optics and the Scanning Electron Microscope

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THE UNUSUAL SHAPE OF THE MAJORITY OF THE RED CELLS in patients with abetalipoproteinemia prompted the use of a separate word to describe these cells. The original description used the word acanthocytosis, but subsequent publications have referred to these cells as acanthocytes. Normal erythrocytes develop regular small spiny protrusions upon their surface when they are washed with isotonic saline. These multiple protrusions disappear when plasma is restored as the surrounding medium for the red cells. Projections or protrusions also appear upon the surface of normal erythrocytes during the early stages of disc-sphere transformation in the course of hemolysis of red cells in a glass chamber. In the present report, normal red blood cells and the red blood cells (acanthocytes) from five patients with abetalipoproteinemia were examined under rigidly standardized conditions by light microscopy using Nomarski optics and with the scanning electron microscope. The effect of changes in the tonicity of the surrounding environment of these cells and of various methods of fixation were also analyzed.

MATERIALS AND METHODS

Subjects were normal healthy adults and five patients with abetalipoproteinemia (M.S., A.V., G.F., D.P. and M.J.). Each patient had been previously studied and the findings reported.

Blood was drawn into plastic disposable syringes and anticoagulated with sodium citrate (0.4 ml. of 20% solution to 9.6 ml. of whole blood). No differences were noted when the anticoagulant was sodium ethylenediamine tetracetate (EDTA) or heparin, or when blood was defibrinated with glass beads. Cells were separated from plasma by gravity (15 minutes at room temperature), though moderate centrifugation on separate aliquots was used to obtain plasma for the preparation of dilute cell suspensions.
In studies with unwashed cells, the red blood cells were diluted with plasma to form a dilute suspension of cells in autologous plasma. Washed red blood cells and washed acanthocytes were obtained by centrifuging whole blood at $3000 \times g$ for 10 minutes. The plasma was then aspirated and replaced by an equivalent amount of physiologic saline, 0.9 per cent. Thorough mixing was achieved by inverting the tube several times and the cells were then allowed to stand for 15 min. at room temperature. Centrifugation was again carried out for 10 minutes. Washing and centrifugation were repeated two additional times. In studies involving changes in the density of the saline, the washed cells, free of 0.9 per cent saline, were suspended in a large volume (hematocrit less than 10%) of either 0.51 per cent saline or 4.25 per cent saline and allowed to stand for 30 minutes before being examined. When the effects of plasma upon saline-washed red cells were studied, the washed red blood cells, free of saline were suspended in plasma (hematocrit less than 10%) for 30 minutes and then studied.

Fixation of the red blood cells for study with the scanning electron microscope was routinely carried out by mixing the red cell suspension (either in plasma or saline) with a five-fold volume of 0.5 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The glutaraldehyde solution was freshly prepared for each study from a stock solution of 25 per cent glutaraldehyde. After standing in the fixative for one hour at room temperature, the cells were washed three times with distilled water during the course of one hour.

For examination in light microscopy, the cells were examined in chambers formed by placing large clean cover slips on cleaned slides. The chamber edges were sealed with heated paraffin. In each chamber, the volume of solution was large in comparison to the number of cells. When fresh plasma was the suspending medium, the cells were examined promptly. A Zeiss binocular microscope fitted with Nomarski optics was used at a magnification of 2000.

Fig. 1.—Preparation of human red cells in sealed chamber (slide and coverslip) photographed with interference microscopy ($\times$ 2000). (A) Red blood cells in native plasma. (B) Red blood cells after three washings in normal saline. Cells are cresated. (C) Red blood cells washed three times in normal saline and suspended in native plasma. Cells are reversed to disc form.
The preparation of the specimens for examination in the scanning electron microscope was as follows: A dilute suspension of the fixed red cells or acanthocytes in distilled water was placed on a circular cleaned coverslip 12 mm. in diameter. The preparation was dried at 37° C for one hour and the coverslip was then cemented to the top of an aluminum support grid. Specimens were coated in an Edwards Vacuum Chamber with gold palladium, and approximately 300 A of metal were deposited on the surface. The specimens were visualized in a Cambridge Stereoscan Electron Microscope at an angle of 45° at 25–30 Kv., and photographs were taken on Ilford film ASA 50.

RESULTS

Interference Microscopy (Nomarski Optics)

Living normal cells in native plasma viewed by interference microscopy appear relatively uniform in shape with a gentle depression delineating the center of the biconcave disc. The surface and edge of the cells are smooth and there is only a slight variation in the diameter of the cells (Fig. 1A). After fixation in glutaraldehyde, the normal cells show some variations in size, but they retain the uniform shape and regularity noted in unfixed preparations. Minimal changes appear to have occurred at the center and edges of the cells, suggesting that neither the fixative nor its buffer creates much distortion (Fig. 2).

Fig. 2.—Examination of human red blood cells after fixation with glutaraldehyde with interference microscopy (× 2000). (A) Red blood cells in native plasma. (B) Red blood cells after three washings in normal saline. Cells are in disc form. (C) Red blood cells washed three times in normal saline and resuspended in native plasma.
Blood from patients with abetalipoproteinemia examined by interference microscopy revealed living acanthocytes with the characteristic large irregular projections from the cell surface of varying size and shape. Frequently the terminal tip of a projection was rounded and enlarged (Fig. 3A). Fixation of acanthocytes with glutaraldehyde did not change the appearance under the Nomarski optics.

The changes in the form of the normal red cells brought about by three washes in normal saline are shown in Fig. 1B. The crenated cells have multiple tiny projections, of fairly uniform size and shape, and the cells appear more spherical then biconcave in shape. Fixation in glutaraldehyde instantaneously returned the normal crenated cell to its previous appearance of smooth biconcave disc indistinguishable from untreated normal cells (Fig. 2B). A variety of concentrations of glutaraldehyde, osmic acid, and formaldehyde all produced the same effect. It was, therefore, not possible to preserve or fix the crenated cell for examination by electron microscopy, either scanning or transmission.

Acanthocytes, after washings in isotonic saline, developed small uniform spicules similar to those found in saline washed normal erythrocytes (Fig. 3B). Fixation of the saline washed crenated acanthocytes with glutaraldehyde, eliminated the crenated appearance (small projections) and the usual form of
the cells was restored. Suspension of these saline washed crenated acanthocytes in autologous plasma, eliminated the tiny spicules caused by saline washings without altering the basic characteristic appearance of the acanthocyte (Fig. 3C). No additional alteration of the shape of the plasma-treated crenated acanthocytes occurred with fixation.

Scanning Electron Microscope

Red blood cells prepared for the scanning electron microscope are fixed in glutaraldehyde, washed with distilled water, dried at 37°C for one hour, and then coated with an alloy of gold palladium in a chamber under high vacuum. Photographs of normal erythrocytes in native plasma resemble in appearance the red cells seen in the light microscope, but the three-dimensional appearance is accentuated. However, the cell rims are more pronounced and the central depressions seem more like holes; the cells can be said to look like doughnuts or life preservers. Not all cells in any field assume these forms. Cells vary considerable in size and shape, possibly due to uneven exposure to fixation and drying, and possibly as a function of the age of the cells (Fig. 4).
Acanthocytes are particularly well visualized in the scanning electron microscope. The three-dimensional appearance shows the many projections from the surface of the cells, and most of the cells have a globular appearance, rather than the normal biconcave disc. Examples of both abnormal and more normal appearing red cells are seen in Figs. 5 and 6.

Changes in the tonicity of the environment before fixation altered the form of the normal red blood cell. Hypotonic saline (0.51%) produced a smooth uniform spherical cell (Fig. 7A) while hypertonic saline (4.25%) produced flattened elongated cells (Fig. 7B).

The alterations in the shape of the acanthocyte caused by changes in tonicity of the environment before fixation are shown in Figs. 8A and 8B. Hypotonicity (0.51% saline) produced a markedly swollen cell, but the large projections of the original cell were still evident, although frequently reduced in extent from the surface of the cells (Fig. 8A). Hypertonic saline (4.25%) flattened the cells and accentuated the projections from the cell surface (Fig. 8B).

DISCUSSION

The morphology of the red blood cells with various technics of study has been extensively described. Unfortunately, the most frequently used method is the examination of the dried, fixed, stained blood smear on a glass slide, in which the preparation of the specimen introduces the greatest number of artifacts, and it is a difficult method to reproduce precisely each preparation. Interference microscopy has the great advantage of examining the red cells in the living state in plasma or other solutions. Nomarski interference optics are an improvement over ordinary phase contrast studies in that the halo about the cells is eliminated and a three dimensional aspect of the cells is shown. The red blood cells can be examined in a relatively large, thick, sealed chamber in which there is a large volume to cell ratio, which minimizes the contact of the red cells with the glass surfaces, and allows for a large amount of plasma which limits the development of crenation. Motion picture photography of blood cells in these chambers has shown that red cells survive with unaltered...
Fig. 7.—(A) Normal red blood cells in hypotonic saline solution (0.51%); scanning electron microscopy ($\times$ 5500). (B) Normal red blood cells in hypertonic saline solution (4.25%); scanning electron microscope ($\times$ 5500).

morphology for hours, and leukocytes and reticulocytes exhibit motion for six to seven hours. The effects of saline washing of red blood cells can be followed in the chamber, and the reversal of the saline effects by restitution of plasma can also be studied. These observations of reversibility of the shape of the erythrocytes signify satisfactory preservation of the integrity of the red cells. Our observations were all made during the initial half hour after chamber preparation, as soon as motion of fluid had sufficiently decreased to permit adequate photography.

The scanning electron microscope has been available for about three years and several publications concerning the appearance of red blood cells with the use of this instrument have appeared. The scanning electron microscope uses a finely focused beam of electrons from a tungsten filament in a vacuum chamber, as in conventional electron microscopy, but the image studied does not depend upon transmission of the electrons but rather upon reflection of the electrons and secondary emissions of electrons from the surface of the specimen. These electrons are collected and amplified and displayed upon the surface of a cathode ray tube and this image can be photographed. Detailed descriptions of the scanning electron microscope can be found in several recent publications.
The method of preparation of the blood specimens for the scanning electron microscope is most important in determining the form and shape of the cells. Fixation with glutaraldehyde is most often used. When the fixed and washed red cells are examined by Nomarski interference optics, little distortion is noted. However, when the fixed washed red cells are examined in the scanning electron microscope after drying and coating with gold palladium alloy, or other metals, changes in size and shape of the cells become apparent (the doughnut appearance). In an effort to avoid these artifacts, some authors have recommended initial freeze drying of the fixed preparation and then subsequent metallization. Studies to develop optimal methods for preparation of blood cells for examination by the scanning electron microscope are being continued. It is obviously important to identify the artifacts created during specimen preparation.

Using Nomarski optics and the scanning electron microscope, a comparison has been made of normal erythrocytes and acanthocytes under varying conditions. Particular attention has been given to the alteration in the shape of the cell membrane. The aberrant shapes seen in acanthocytes in autologous plasma in light microscopy are preserved in glutaraldehyde fixation and are well
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visualized in the Nomarski optical system. The large projections from the cell surface remain after drying and metallization in vacuo and are seen in the photographs taken with the scanning electron microscope.

The capacity of the red blood cell membrane to change its shape depending upon the tonicity of the environment is a feature of both the normal erythrocyte and of the acanthocyte. The altered volume of the cell, presumably as a function of the water content of the cell, results in a different shape, as illustrated in Figs. 7 and 8. These changes are reversible, if certain limits of tonicity are not exceeded.

Projections from the surface of the red cell are the characteristic feature of the acanthocytes in patients with abetalipoproteinemia. Although first described almost 20 years ago, the explanation for the abnormal shape is not yet available. Although certain chemical differences in the lipid composition of the red cell membrane have been observed in acanthocytes compared with the composition of normal red cells, the relation of these differences to abnormal shapes is obscure. During the past years a number of patients, in whom the plasma betalipoprotein content was normal but who have spicules on their red cells have been reported. These reports include patients with pyruvate kinase deficiency, thrombotic thrombocytopenic purpura, microangiopathic hemolytic anemia, uremia and carcinoma.14-18 Most of these reports are based on the examination of the pulled dried blood smears and it would be of interest to examine these cells by Nomarski optics and scanning electron microscopy.

Recently, the red blood cells of a small number of patients with cirrhosis have been described as spurred.19-22 These patients have anemia, an elevated level of bilirubin, a considerable degree of hemolysis with shortened red cell survival time, and their plasma can, in some cases, induce spurting of normal red cells.

It is suggested that the word acanthocyte be reserved for the red cells found in patients with abetalipoproteinemia. Descriptive words such as burr cells and spurred cells should be coupled with the primary disease state in which they are found.

**Summary**

The morphology of normal erythrocytes and of acanthocytes from five patients with abetalipoproteinemia was studied by two relatively new technics. Interference microscopy with Nomarski optics was used to study the red blood cells in the living state; the scanning electron microscope was utilized to give a three dimensional appearance of the fixed red blood cells.

Isotonic saline washing of normal red blood cells produces multiple small uniform projections on the surface of the cell: the crenated red cell. Acanthocytes after saline washings also develop similar projections: the crenated acanthocyte. Crenation of both the normal red blood cell and the acanthocyte is reversed by restitution of a plasma environment and by chemical fixation by glutaraldehyde or osmic acid but the characteristic shape of the acanthocyte is preserved.

A comparison was made of the changes in the shape of the normal red blood
cells and acanthocytes induced by alterations in the tonicity of the surrounding medium.

REFERENCES
