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The Sub-Membrane Reticulum of the Human Erythrocyte: A Scanning Electron Microscope Study

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A web-like reticulum underlying the human erythrocyte membrane was studied at a resolution of 5–10 nm by means of a scanning electron microscope. The network was visualized in isolated membranes (ghosts) torn open to reveal their interior space and in residues derived from ghosts extracted with Triton X-100. It formed a continuous (rather than patchy) cover over the entire cytoplasmic surface, except where lifted off or torn away. Filaments (5–40 nm in diameter), annular figures (40–60 nm in diameter), and nodes (30–100 nm in diameter) were prominent in different networks. The dimensions of the filaments and the interstices in the reticulum varied with conditions, suggesting that the network has elastic properties. This reticulum is probably related to the erythrocyte membrane proteins spectrin and actin.

Key words: red cell, erythrocyte, membrane, scanning electron microscope, spectrin, actin

Marchesi and his associates first described a water-soluble protein fraction selectively eluted from erythrocyte membranes in low ionic strength buffer. The addition of Ca^{2+} and the elevation of the ionic strength of this extract induced the appearance of 4–6-nm diameter filaments of variable length (1). This material was termed spectrin (1). Subsequent electrophoretic analysis has shown that such preparations contain 3 major polypeptides: bands 1 and 2 (apparently of 240,000 and 215,000 daltons, respectively) and band 5 (molecular weight ~ 43,000) (cf. Ref. 2). Most investigators now limit the term spectrin to bands 1 and 2, while band 5 has the attributes of actin (3, 4).

Ferritin-conjugated antibodies (5, 6) as well as enzymic and chemical probes (2, 7–9) have shown that both spectrin and actin are associated with the inner (cytoplasmic) surface of the red cell membrane. Spectrin has been equated with irregular patches of fibrillar material adherent to the cytoplasmic surface of the isolated erythrocyte membrane, as visualized in electron micrographs of thin sections of ghosts (7, 10). Furthermore, Triton X-100 selectively extracts most of the lipids and integral membrane proteins from ghosts under conditions where spectrin and actin remain insoluble (11). The residues surviving this detergent extraction appeared as ghost-shaped matrices of densely packed, self-associated filaments, both by negative staining and by the thin section electron microscopy (11, 12). A possible skeletal role for such a submembrane reticulum is suggested by the mechanical properties of intact red cells and isolated ghosts, and particularly by changes in deformability resulting from the depletion of cellular energy sources (9, 13). Some authors

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have also suggested that the spectrin system may constrain the lateral motion of membrane-integrated proteins (14, 15).

Despite these observations, the ultrastructure of this supramolecular array in situ remains unclear; in fact, the very existence of filaments involving spectrin and actin in the intact red blood cell has been questioned (3). High resolution scanning electron microscopy offers a valuable approach to the elucidation of the surface architecture of membrane-associated structures (cf. Ref. 16) and has therefore been used in this study to gather new data on the submembrane reticulum of the red cell membrane. Brief accounts of this research have been presented (17, 18).

MATERIALS AND METHODS

Red Blood Cells

Human blood from normal donors was collected in 10 mM EDTA (final) as in Ref. 19. In addition, blood stored for 3–4 weeks in citrate-phosphate-dextrose was obtained from the University of Chicago blood bank. The intact erythrocytes were washed extensively in saline (150 mM NaCl/5 mM sodium phosphate, pH 8) as in Ref. 19.

Washed red blood cells were affixed to supports in 2 ways. In the first, a drop of cells ($\sim$ 25 μl) was simply applied to a clean, untreated aluminum disk for 1 h. (Shorter application periods reduced cell attachment.) In the second method, disks were pretreated with a drop of 10^{-3} M polylysine (molecular weight $\sim$ 2,000; Sigma Chemical Company, St. Louis, Missouri) for 10 min and rinsed several times in saline before application of the cells for 15 min. [Other investigators have also reported on the utility of polylysine in affixing membrane systems to supports for scanning electron microscopy (20, 21).] The substrate disks were maintained throughout in shallow plastic dishes on ice. After cell attachment, the disks were rinsed by filling the dish with 4 ml of cold saline, then transferring the specimens to 2 fresh dishes of iced saline.

Ghosts and Residues

To generate ghosts, disks bearing red blood cells were immersed in ice-cold 5 mM sodium phosphate buffer (pH 8.0) for 1–2 min and then rinsed well with fresh buffer until all traces of red were removed. Lysis and washing took less than 5 min. (We took these ghosts to be free of cytoplasm, since retention of 1% cellular hemoglobin or less imparts a distinct red color.)

Detergent-extracted membrane residues were prepared by immersing disks bearing ghosts for 30 sec in an ice-cold solution of 0.2% Triton X-100 (Sigma) in 5 mM sodium phosphate, pH 8.0 (cf. Ref. 11). The samples were then washed twice in the same chilled buffer without detergent.

Scanning Electron Microscopy

Fixation was effected by exposing samples for 30 min to 0.5% buffered glutaraldehyde solutions (derived from an 8% stock from Polysciences Incorporated, Warrington, Pennsylvania) and then washing thoroughly with deionized water, as above. Specimens were rapidly frozen by immersion of the disks in a Freon bath cooled with liquid nitrogen. Disks were rapidly transferred to an insulated copper block at liquid nitrogen temperature for freeze-drying. (This block stood on 3 conical stainless-steel legs, whose sharply-pointed tips minimized heat transfer.) The copper block was placed in an Edwards 306 vacuum

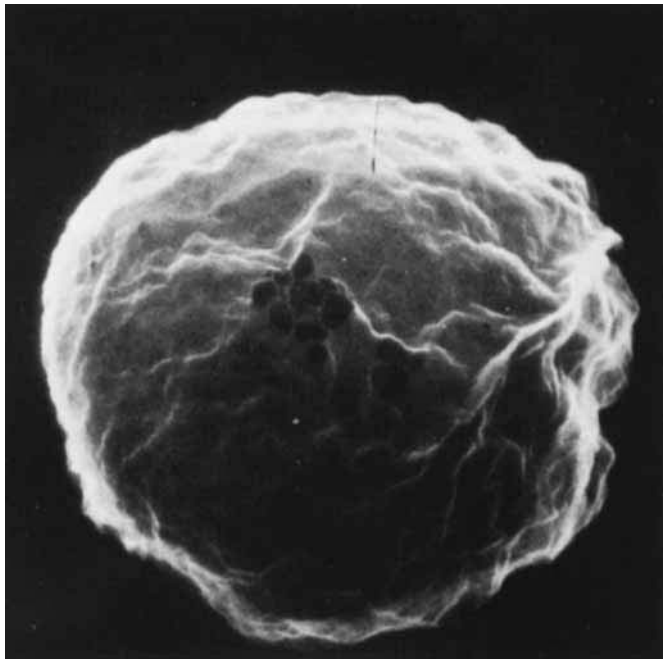


Fig. 1. A scanning electron micrograph of a nonflattened ghost. Washed red blood cells were affixed to an aluminum disk without polylysine and lysed in situ. The specimen was fixed in glutaraldehyde, rinsed, freeze-dried, and coated as described in Materials and Methods. Magnification 10,000 ×.

evaporator equipped with a liquid nitrogen trap (Edwards High Vacuum, Inc., Grand Island, New York). In the morning, the specimens were slowly warmed from approximately -20° to room temperature with a heating element placed under the block. (The temperature was monitored by a thermocouple in the center of the block.) This step prevented the condensation of moisture when the vacuum was released.

In preliminary experiments, we found that glass supports were inferior to aluminum disks. Similarly, critical point drying was suboptimal, because a considerable loss of membranes from the supports occurred in each washing step, and the use of an organic solvent risked extraction of membrane material and distortion (cf. Ref. 21).

The disks were rotary-tilt coated with 5–10 nm of Au/Pd (60:40), from a distance of 10 cm and at an angle of 45°, in the Edwards 306 evaporator. Specimens were examined with a field emission Hitachi HFS-2 scanning electron microscope, operated at 25 kV and emission current of 1.5–2.0 μA, under a vacuum of 5×10^{-8} torr.

RESULTS AND DISCUSSION

Experiments were carried out on both freshly-drawn and outdated red blood cells. The results were similar in both cases. However, few ghosts from fresh cells flattened and tore open, so that most of our micrographs were obtained from outdated cells; hence, their presentation below.

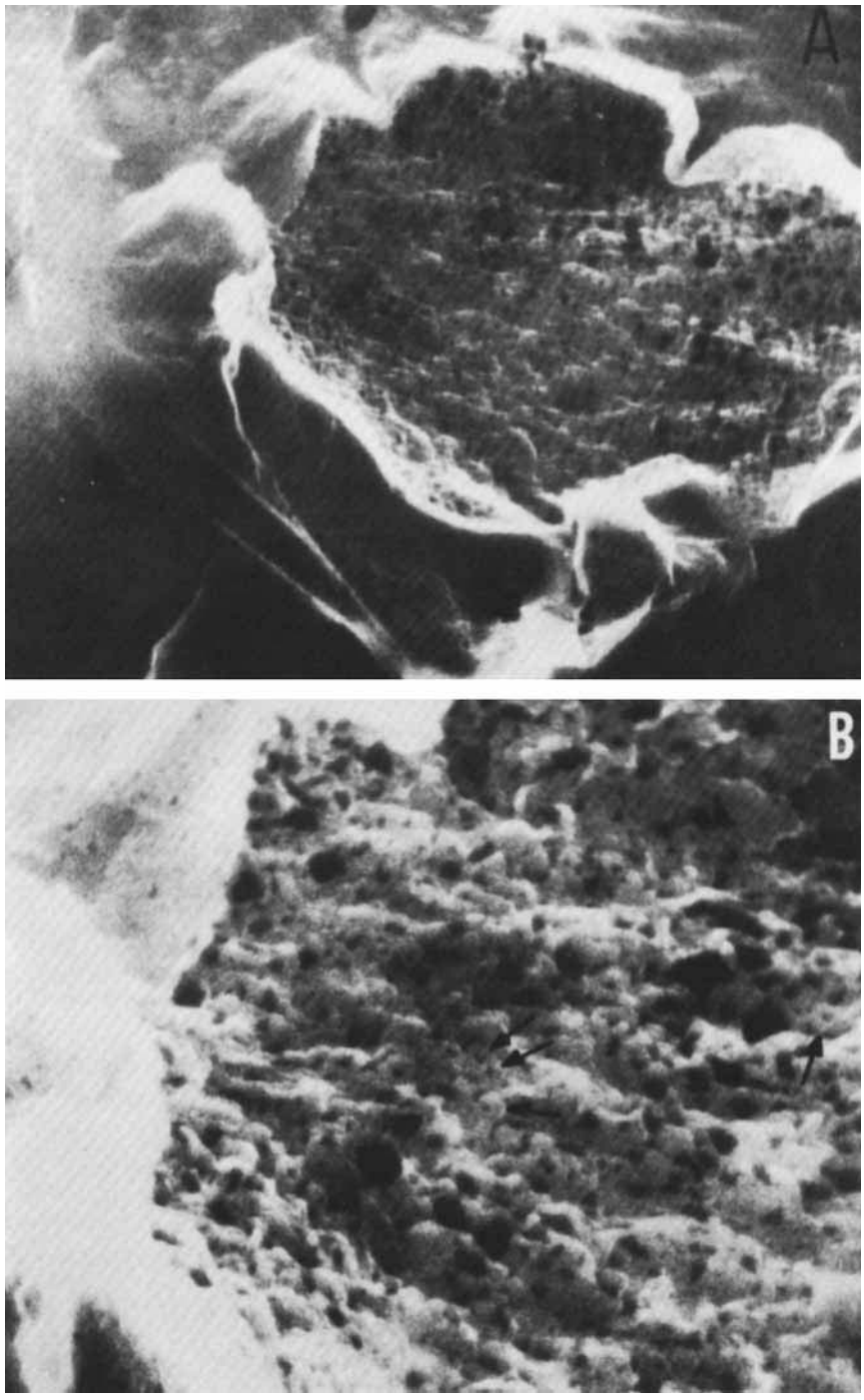


Fig. 2. The reticulum in a torn, nonflattened ghost. Red blood cells were processed as in Fig. 1. A) A layer of the dome of this ghost has apparently been removed, revealing the superficial surface of an underlying reticulum. Magnification 20,000 ×. B) A portion of this ghost at higher magnification. Arrows indicate a few of the many annular figures. Note the polygonal shapes of the larger holes and occasional appearance of individual processes (filaments). Magnification 50,000 ×.

Ghosts on Supports Lacking Polylysine

Intact erythrocytes on disks with and without a polylysine coating were examined by scanning electron microscopy. These structures (not shown, but see Ref. 17) resembled the biconcave disks seen in numerous studies (23).

Ghosts from red cells affixed to supports in the presence and absence of polylysine were examined. In the absence of polylysine, the ghosts were typically globoid (rather than flattened), with wrinkled surfaces (Fig. 1); they were considerably less distorted however, than the ghosts visualized in a previous scanning electron microscope study (24). Round dark patches, often in clusters, were frequently observed on the ghost surfaces. Since these structures were not observed in preparations of intact erythrocytes or sealed vesicles, they could represent holes caused by hypotonic hemolysis, such as previously studied by Seeman et al. (25).

Infrequently, ghosts appeared to have a portion of the dome of the membrane torn away, as shown in Fig. 2A. This lesion exposed a subjacent meshwork which appeared to extend across the defect. We may thus be viewing the superficial surface of a submembrane reticulum, as shown schematically in Fig. 3B.

Figure 2B shows this reticulum at higher magnification. The dominant elements were closely-spaced annular figures of 40–60-nm diameter, with central holes of approximately 20-nm diameter. Larger (60–140 nm) and frequently polygonal holes were also observed between the annuli.

The annuli seen in Fig. 2B could correspond to the hollow cylinder, "torus" protein described by Harris (26, 27) in low ionic strength extracts of ghosts. The rings seen in our study, however, appeared to significantly larger than the reported 13.5-nm diameter toruses. These forms might also relate to the "C" shaped structures of ~ 30-nm diameter found by Tilney and Detmers (3) in EDTA extracts containing unpolymerized spectrin.

Ghosts on Polylysine-Coated Supports

Analogous studies were performed using disks treated with polylysine. Flattened ghosts were particularly prevalent in these preparations. Almost all of these collapsed membranes had torn open, presumably as a result of stresses induced by adhesion to the underlying polycationic surface (Figs. 4 and 5). Ruptured ghosts showed 2 distinct morphologic patterns; the first is illustrated in Fig. 4A. Four regions may be noted: α, a smooth outer apron, reflecting the flattened external surface of the membrane; β, the margin of the tear itself; γ, a region internal to the torn edge, having the appearance of a draped webbing; and δ, the floor of the ghost, covered with a reticulum similar to that seen in greater relief in the raised γ region.

The γ reticulum is continuous with that covering the δ and ϵ regions, but appears to be lifted off the membrane. It may have been released from the flattened and recessed perimeter of the ghost (α) so as to form a drape screening the mouth of that pocket (as in Fig. 3C).

The reticulum is viewed at high magnification in Fig. 4B. Filaments approximately 5–10 nm in diameter are associated with the floor of the ghost (δ). (Since the resolution of this microscope is approximately 5 nm, smaller processes would not be resolved here.) The raised (γ) region is comprised of filaments 5–40 nm in diameter, as well as infrequent annular forms. The "holes" in the γ network vary between 20 and 100 nm, while those in the δ region are generally larger. Figure 4 suggests that the processes in the δ region are stretched out, presumably because of their adherence to the subjacent flattened membrane, while the γ filaments may be condensed or contracted.

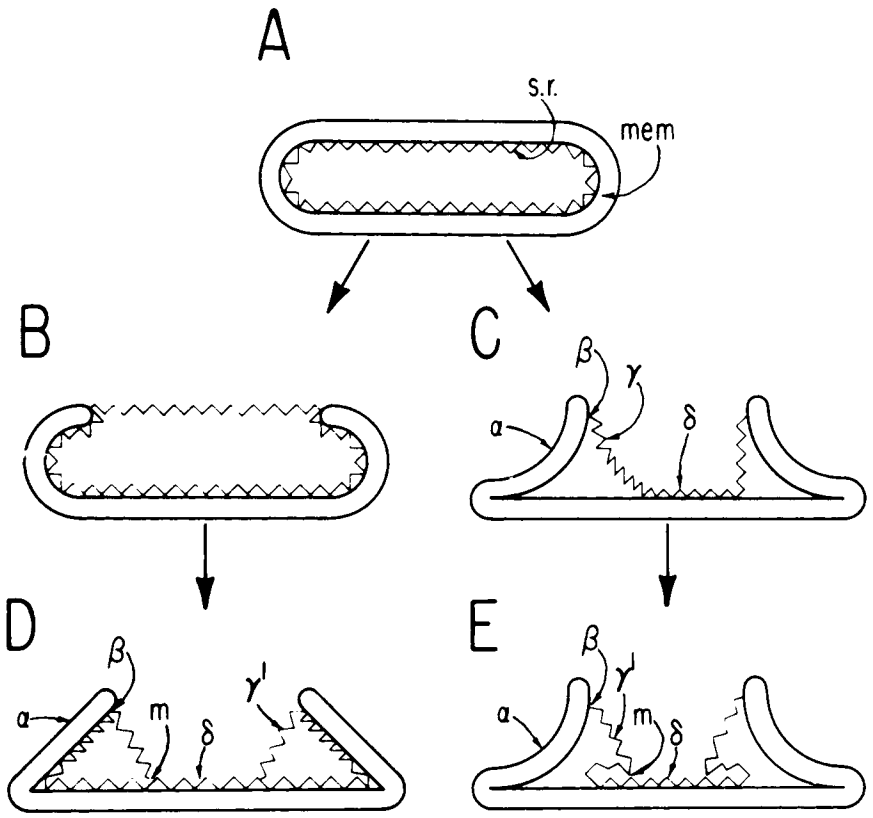
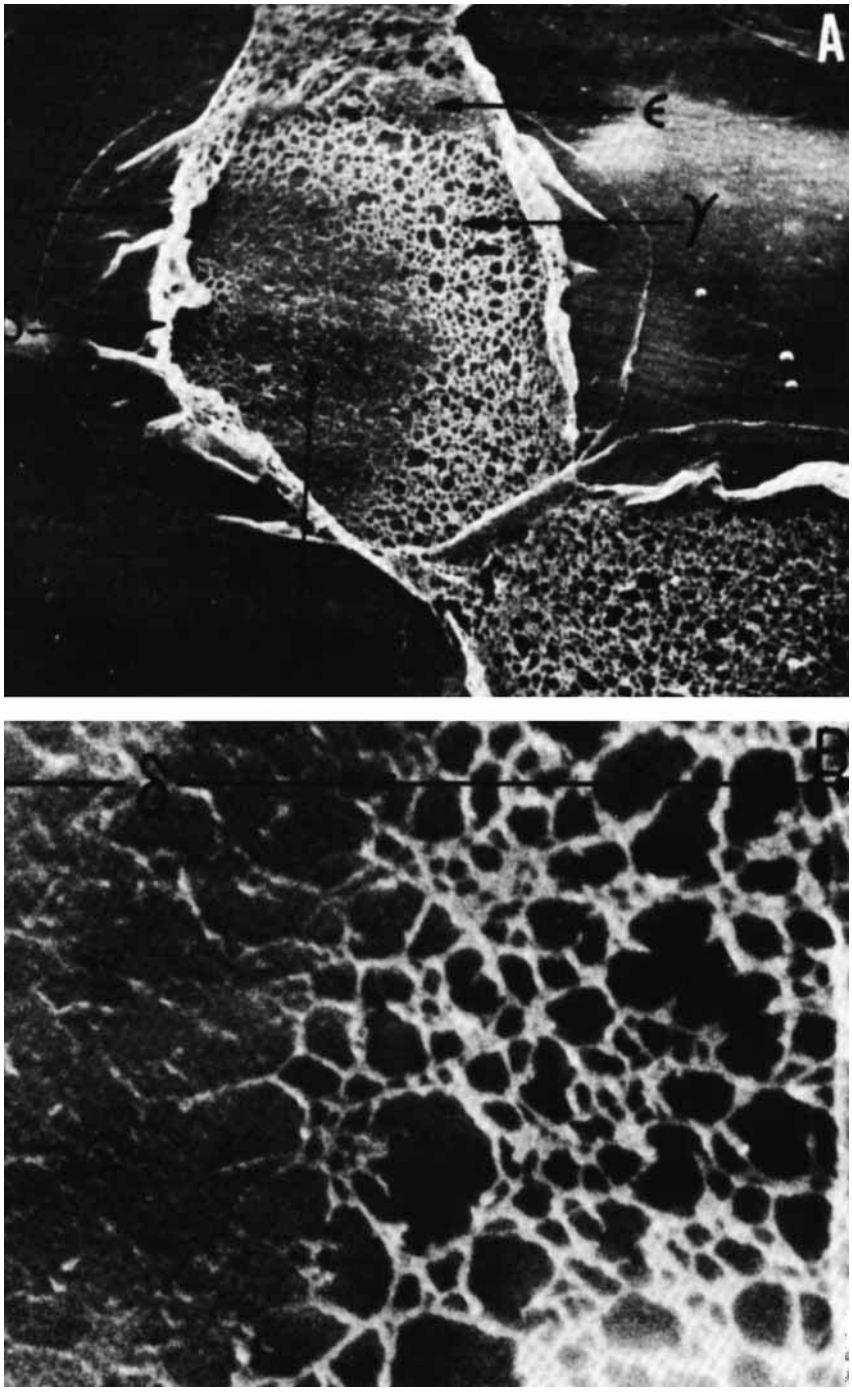


Fig. 3. Possible interpretations of the structures seen in torn ghosts. (Drawings are saggital cross sections; Greek letters correspond to designations in Figs. 4 and 5.) A) The intact ghost attached to substrate; mem, membrane layer; s.r., submembrane reticulum. B) The dome of the ghost has torn, exposing the subjacent reticulum (compare Fig. 2A). C) The dome of a flattened ghost has torn away with submembrane filaments attached. The lateral portion of the remaining reticulum (γ) has separated from the inner surface of the flattened membrane apron (α) (compare Fig. 4A). D) If the superior sub-membrane meshwork in scheme B tears, this reticulum (γ') might fall onto the inner membrane surface (δ) creating the observed discontinuous margin, m (compare Fig. 5). E) Alternatively, the relaxed lateral reticulum (γ) in scheme C may roll in upon the basal meshwork (δ) to create a redundant fold, appearing as a discontinuous margin, m (compare Fig. 5).

Fig. 4. A continuous reticulum in a torn, flattened ghost. A) This specimen was prepared as in Fig. 1 and 2, except that the aluminum disk was precoated with polylysine. Two adjacent, flattened ghosts have torn open. Five distinct regions are revealed: α, the outer surface of the membrane; β, the torn margin; γ, a nonadherent (raised) filamentous web; δ, the inner (cytoplasmic) surface of the floor of the ghost; and ϵ, a raised portion of the inner surface of the membrane. Note that both the δ and ϵ surfaces are covered with filaments in continuity with the γ region. Magnification 10,000 ×. B) A portion of the transition zone between the γ and β region in another ghost. Magnification 50,000 ×.


A
ε
γ
B

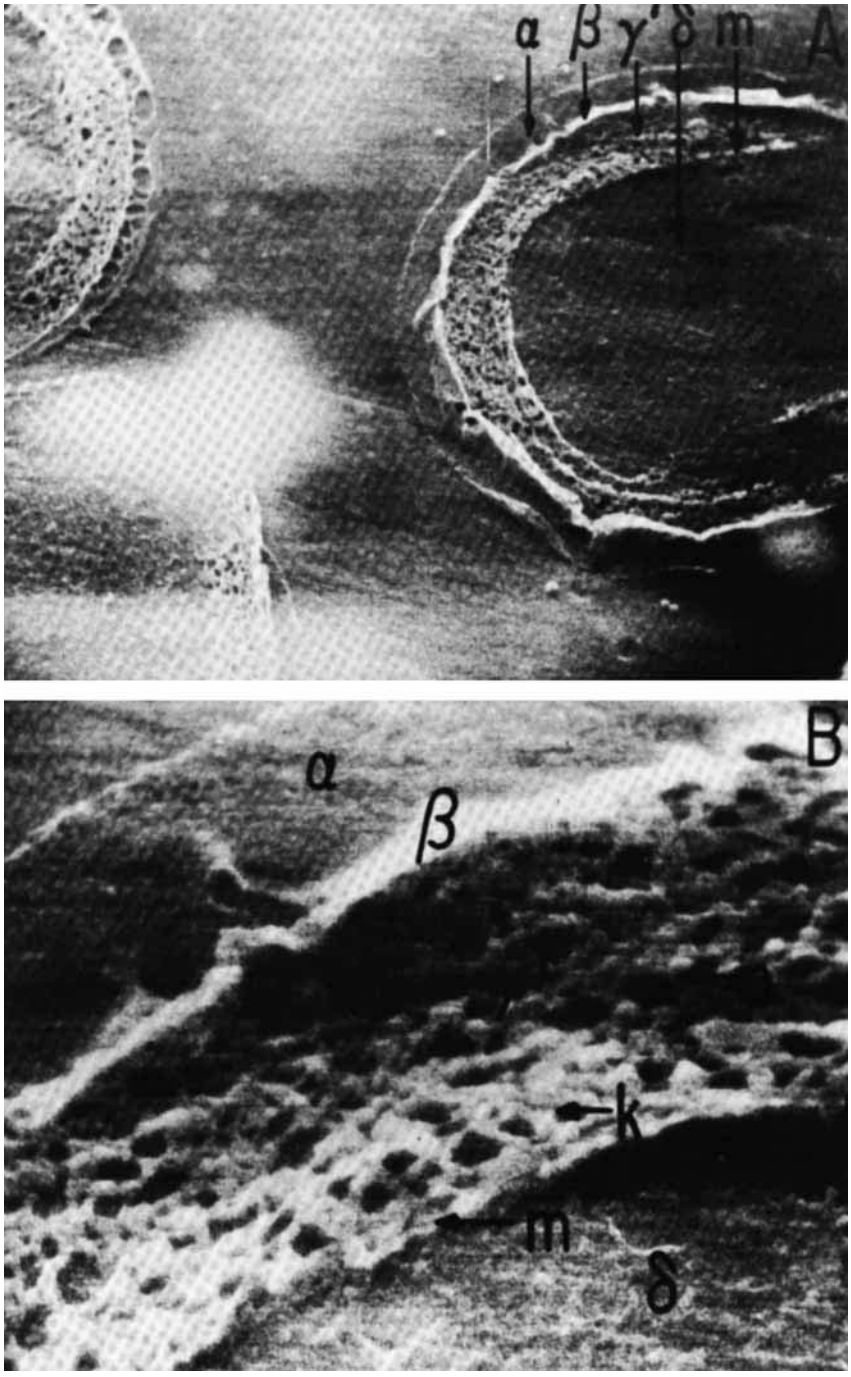


Fig. 5. A discontinuity in the reticulum of a torn, flattened ghost. This specimen was the same as in Fig. 4, the 2 fields being separated by a few millimeters. A) The labeled areas correspond to those in the preceding figure: α, outer surface of the ghost; β, its torn edge; γ', a raised web; δ, the inner surface of the floor of the ghost. Note that the regions γ' and δ appear separated by a discontinuous margin, m. Magnification 10,000 ×. B) A portion of the labeled ghost in panel A). Note the appearance of knobs (k) and a pentagonal hole (p) within the γ' region. Magnification 50,000 ×.

On polylysine-coated disks, the reticulum frequently extends beyond the perimeter of the membrane from which it arises. In Fig. 4A, we see the meshwork projecting above and below the ghost. It is conceivable that a highly polyanionic network (e.g., of spectrin plus actin) might creep along the polycationic disk surface. In rare instances, we have observed a whole network thrown free of its parent membrane or merely tethered to it (17). Such images strengthen the hypothesis (2, 11) that the reticulum is held together by self-association and does not depend on the rest of the membrane for its integrity. These findings do not support the postulated existence of tight and extensive binding between the reticulum and membrane-integrated proteins (14, 15).

Torn ghosts assumed a second principal configuration, illustrated in Fig. 5. In this case, a discontinuity (the margin marked m) exists between the networks of regions γ' and δ. Two interpretations of this discontinuity are considered. The membrane may first tear, leaving the submembrane reticulum draped across the gap (Fig. 2 and Fig. 3B). If the center of this webbing were to tear and the torn web were to drop onto the inner membrane surface, its edge would create this margin, as depicted in Fig. 3D. Alternatively, it is possible that the discontinuity is caused by the rolling back upon itself of a continuous, relaxed, and redundant webbing; this interpretation is shown schematically in Fig. 3E.

The γ' reticulum in Fig. 5B resembles that seen in Fig. 2, appearing more condensed or contracted than the γ region in Fig. 4. Figures 2, 4, and 5 suggest that the reticulum has elastic properties, being stretched out when flattened on polylysine and relaxed when free. Elastomeric material in the red cell membrane has, in fact, been postulated on the basis of theoretical (28, 29) and experimental (13) studies on erythrocyte deformability. Other variations in the morphology of the meshwork (e.g., the annular figures, knobs, etc.) also may reflect plasticity in the disposition of the elements in this reticulum. Occasionally, a single torn ghost may exhibit all 3 distinct reticular patterns: an extended network of filaments, a contracted network of filaments, and zones of annular figures. The submembrane structures are not a function of residual cytoplasmic material, since the same results were obtained using hemoglobin-free ghosts prepared in bulk according to Fairbanks et al. (19).

Ghosts Extracted With Triton X-100

Treatment of ghosts with Triton X-100 solubilized most of the membrane lipids and membrane-intregated proteins, leaving insoluble ghost-shaped residues rich in spectrin and actin (11). In the present study, a 30-sec exposure to Triton X-100, followed by rapid rinsing and fixation, caught some ghosts in the midst of the extraction process. These structures contained masses of small blebs and smooth vesicles, apparently ghosts on their way to dissolution (not shown). Other ghosts, however, were fully extracted at this time. As seen in Fig. 6, their morphology was reminiscent of the reticular patterns demonstrated in Figs. 2, 4, and 5, and of the Triton X-100 residues previously studied by negative staining and thin-section electron microscopy (11, 12).

The close similarity between the reticulum in ghosts and in Triton X-100 residues (compare Fig. 6 with Fig. 4A) encourages the view that these systems are essentially the same. Since Triton X-100 residues are known to contain a preponderance of spectrin and actin (11), the webs seen in the unextracted, ruptured ghosts are also likely to contain these components. Further support for this premise was obtained by examining preparations rich in inside-out vesicles. These inverted vesicles would be expected to bear spectrin and actin (polypeptide bands 1, 2, and 5) on their exposed surface, were it not that the low ionic strength treatment employed in their generation selectively elutes these proteins

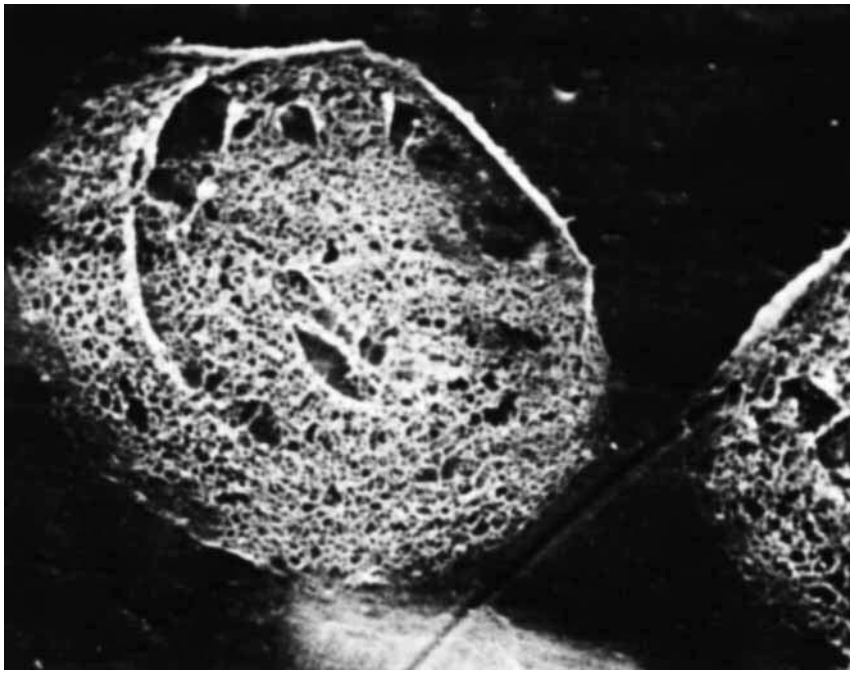


Fig. 6. A ghost residue following Triton X-100 extraction. Washed red blood cells were affixed to an aluminum disk coated with polylysine and converted to ghosts by hemolysis in situ. The support was than treated for 30 sec with 0.2% Triton X-100, then rinsed, fixed, freeze-dried, and coated as described above. Magnification 10,000 ×.

from the membrane (cf. Ref. 30). In the scanning electron microscope, these vesicles did not tear open, but exhibited wrinkled and folded surfaces, much like the parent ghost shown in Fig. 1. As predicted, they lacked any evidence of filaments or reticular networks.

CONCLUDING REMARKS

We have observed in a high-resolution scanning electron microscope a continuous reticulum at the inner surface of ghosts and in the residues derived from Triton X-100 extracted ghosts. We have inferred that spectrin and/or actin are major components of this system, although other proteins are also likely to be present. The finding of these structures was entirely expected, since they are in accordance with a wealth of previous data (see Introduction). What is striking is the degree of structural detail afforded by this scanning electron microscopy technique and the variety of configurations the reticulum assumes under different conditions.

Future scanning electron microscope studies should attempt a positive identification of the structures observed here; the use of conjugated antibodies to purified membrane proteins (as in Refs. 5 and 6) would be one example. A systematic exploration of the polymorphism of the meshwork is also warranted. It is unlikely that the structures observed here precisely represent the native disposition of the reticulum, particularly since we primarily utilized red cells aged in vitro. Future studies should endeavor to relate scanning electron microscope data to various physiologic, pathologic, and experimentally-induced states of intact erythrocytes, alterations in cell shape being one obvious example.

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