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Mechanism of Red Blood Cell Acanthocytosis and Echinocytosis *in Vivo*

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Summary. Patients with abetalipoproteinemia have an inborn absence of the major apoprotein of low density plasma lipoproteins, an abnormal serum and red cell lipid profile, and spiny erythrocytes, called acanthocytes. We now show that these deformed cells are reversibly converted to a normal shape, that of a biconcave disk, by incubation with 3 to 10×10^{-5} M chlorpromazine. We suppose that chlorpromazine acts by expanding the cytoplasmic leaflet of the bilayer, thus promoting inward curvature. Ghosts isolated from the acanthocytes are themselves spiny but are also converted to normal, convave disks by chlorpromazine or merely by a brief incubation at 37 °C in low ionic strength buffer. We attribute the latter to a redistribution of lipids between the two leaflets of the membrane bilayer. Similar observations were made with red cells and ghosts from a patient with benign echinocytosis. These observations suggest that the morphological abnormality in acanthocytes and echinocytes can be ascribed to the same mechanism as crenation *in vitro;* that is, a bilayer couple effect in which an excess of surface area in the outer leaflet over the inner leaflet of the membrane bilayer drives outward curvature. It is striking that cells which were chronically abnormal in shape *in vivo* contain the information to become biconcave disks immediately upon simple chemical treatment *in vitro*.

Key Words erythrocyte · cell shape · bilayer · acanthocytosis · echinocytosis · chlorpromazine

Introduction

The form of the human erythrocyte, a biconcave disk, is a classical starting point for inquiry into the determinants of cell shape. A variety of human disorders associated with misshapen red cells provides an important approach to the problem [4, 16].

Abetalipoproteinemia results from a congenital absence of apoprotein B, the major polypeptide of plasma low density lipoproteins; the reduced lipid-carrying capacity of the plasma leads directly to many facets of the disease [5, 13]. Erythrocytes from these patients have a high-normal or slightly elevated membrane cholesterol and an elevated ratio of sphingomyelin to phosphatidylcholine [5, 8]; however, it is uncertain whether these changes in membrane composition cause the spiny protuberances which give the diseased red cells their characteristic acanthocytic shape [3, 7].

There is considerable debate and conjecture concerning the extent to which membrane proteins (a submembrane network of spectrin and actin in particular) and membrane lipids determine red cell contour (*see* refs. 14 and 15 for review). The bilayer couple hypothesis attributes control of membrane curvature to the unequal surface area of the two leaflets of the bilayer [10, 21]. Experimentally, agents which are believed to expand the outer surface differentially (e.g. the dinitrophenolate anion) cause evagination: crenation and echinocytosis. Agents believed to expand the cytoplasmic surface differentially (e.g. the chlorpromazine cation) cause invagination: pocking, endocytosis, cupping or stomatocytosis [9, 21].

If the projections characteristic of the acanthocytes in abetalipoproteinemia are caused by an excess in the area of the outer leaflet of the bilayer secondary to equilibration with the abnormal lipid contents of the plasma, the altered shape might be rectified by expanding the cytoplasmic leaflet. Testing this hypothesis is the thrust of this report.

Materials and Methods

Freshly drawn whole blood from male and female siblings with previously diagnosed abetalipoproteinemia and two normal controls was provided by Samuel Lux of the Children's Hospital Medical Center, Boston, Mass. (In all respects tested, the two controls and the two acanthocytic blood samples were indistinguishable and were therefore treated interchangeably in this report.) On another occasion, a fresh sample of blood from a healthy donor with a benign idiopathic echinocytosis was provided by Patrick Ward of the Mount Sinai Hospital, Minneapolis, Minn. The samples were drawn into 10 mM Na_4EDTA, stored on ice, and promptly flown to Chicago. Experiments were begun on the day following venipuncture.

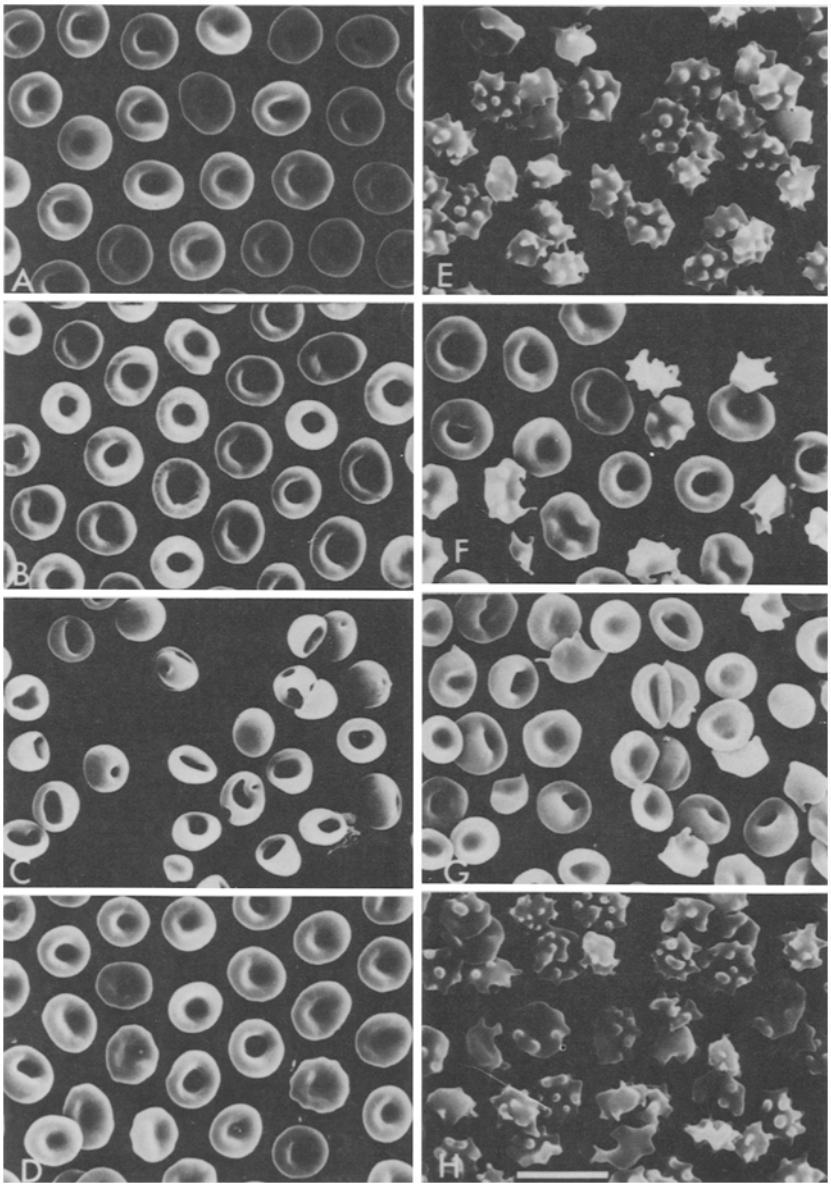

Fig. 1. Effects of chlorpromazine on the contour of erythrocytes from patients with abetalipoproteinemia. Normal (left) and patient (right) red cells were washed in 0.15 M NaCl-5 mM NaPi (pH 8) and suspended in seven vol of the same buffer containing, 0, 0.03 or 0.1 mM chlorpromazine on ice. After 5 min, the suspensions were made 1% in glutaraldehyde. Cell morphology was examined both by dark-field light microscopy and scanning electron microscopy (shown here). For scanning, the fixed cells were washed twice in deionized water, spread on glass slides, and air dried at ambient temperature. The sample was coated with gold-palladium (60:40) and photographed at 2000× in a JEOLCO TSM-U3 microscope operated at 25 kV with a stage angle of 20°. Panels *A–D*, normal cells; panels *E–H*, abetalipoproteinemic red cells. Chlorpromazine (mM): 0 (panels *A* and *E*); 0.03 (panels *B* and *F*); 0.1 (panels *C*, *D*, *G* and *H*). In panels *D* and *H*, the cells treated with 0.1 mM chlorpromazine were washed thrice in buffer to free them of chlorpromazine prior to fixation. Calibration bar = 10 μm

Red cells were washed in saline and ghosts prepared in 5 mM NaPi (pH 8.0) as described [11]. All procedures were performed on ice unless noted. Further experimental details are given in each Figure legend.

Results

Acanthocytes: Intact Cells

Upon arrival, samples of the untreated whole blood were inspected by dark-field microscopy. The red cells of normal donors were a uniform dispersion of biconcave disks with only rare spiny cells. (Mild echinocytosis is a common artifact of blood storage.) Cells from the two patients with abetalipoproteinemia were not biconcave but were distorted in two ways. Many cells had several regular, broad projections and resembled echinocytes (although echinocytes generally have more and narrower spicules – e.g. *see* Fig. 3 below). A small number of cells had an irregular contour with a few bizarre, slender projections, characteristic of acanthocytes [3–5, 13]. Many cells had a form intermediate between the two extremes, and normal cells were rare. Ultracentrifugal analysis of the plasma from these samples demonstrated a lack of low density lipoproteins, confirming the diagnosis. Furthermore, the lipids in these red cell membranes showed a mild elevation in cholesterol/phospholipid ratio and an increase in the sphingomyelin/phosphatidylcholine ratio, both charac-

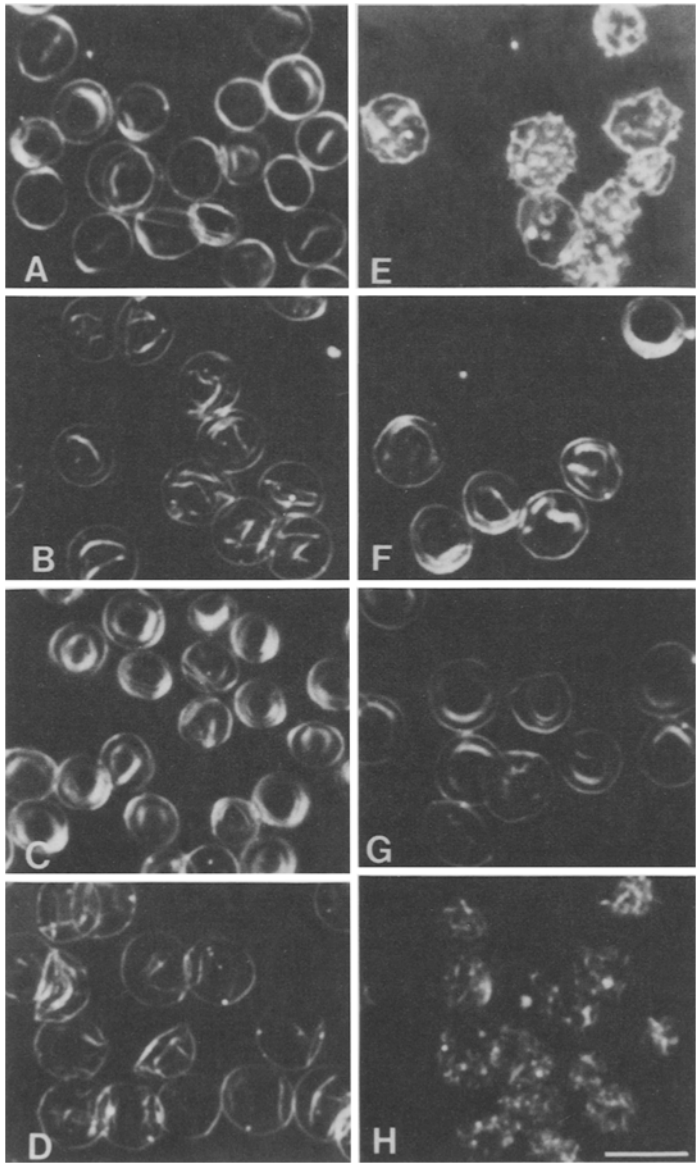

Fig. 2. Manipulation of the contour of ghosts from patients with abetalipoproteinemia. Experiment I: Ghosts from normal (left) and patient (right) red cells were prepared in 5 mM NaPi (pH 8)-0,01 mM $MgSO_4$ as described [11, 14], and suspended in seven vol of 5 mM NaPi (pH 7.5) containing 0 (panels *A* and *E*) or 0.1 mM chlorpromazine (panels *B* and *F*) on ice. After 5 min, the suspensions were fixed in 1% glutaraldehyde. Experiment II: Ghosts were prepared in 5 mM NaPi (pH 8) and warmed at 37 °C for 3 min in seven vol of the same buffer, then chilled on ice before fixation (panels *C* and *G*). In panels *D* and *H*, the samples were brought to 0.15 M NaCl prior to fixation. Specimens were photographed in a Nikon dark-field microscope at 1000× magnification. Calibration bar = 10 μm

teristic of the disease [5, 13]. Thus, while these patients had an unusually high fraction of echinocytes and low fraction of classical acanthocytes, they seem otherwise representative of the disease.

The effect of chlorpromazine on the contour of washed normal and abnormal cells was then evaluated by dark-field microscopy and confirmed by scanning electron microscopy. Control cells were uniformly biconcave disks, while the patients' cells were distorted by multiple projections over their entire surface (Fig. 1, panels *A* and *E*). (These and all subsequent micrographs are representative of the many highly homogeneous fields examined.) No change in the normal cells was observed in the presence of 0.01 mM chlorpromazine or less, while in the diseased population, many of the cells were converted to an approximately normal contour (*not shown*). Chlorpromazine at 0.03 mM induced a more deeply dimpled contour in normal red cells (Fig. 1 *B*), while 0.1 mM caused both deep cupping and small endocytic invaginations (Fig. 1 *C*). These features are characteristic of stomatocytes [4]. Chlorpromazine at 0.03 mM reduced the frequency and magnitude of projections seen in the diseased red cells, converting many of them to dimpled disks similar to normal cells (Fig. 1 *F*). In 0.1 mM chlorpromazine, the diseased red cells lost their projections almost completely, and many became mildly stomatocytic (Fig. 1 *G*). As suggested to us by an astute reviewer, the cells with

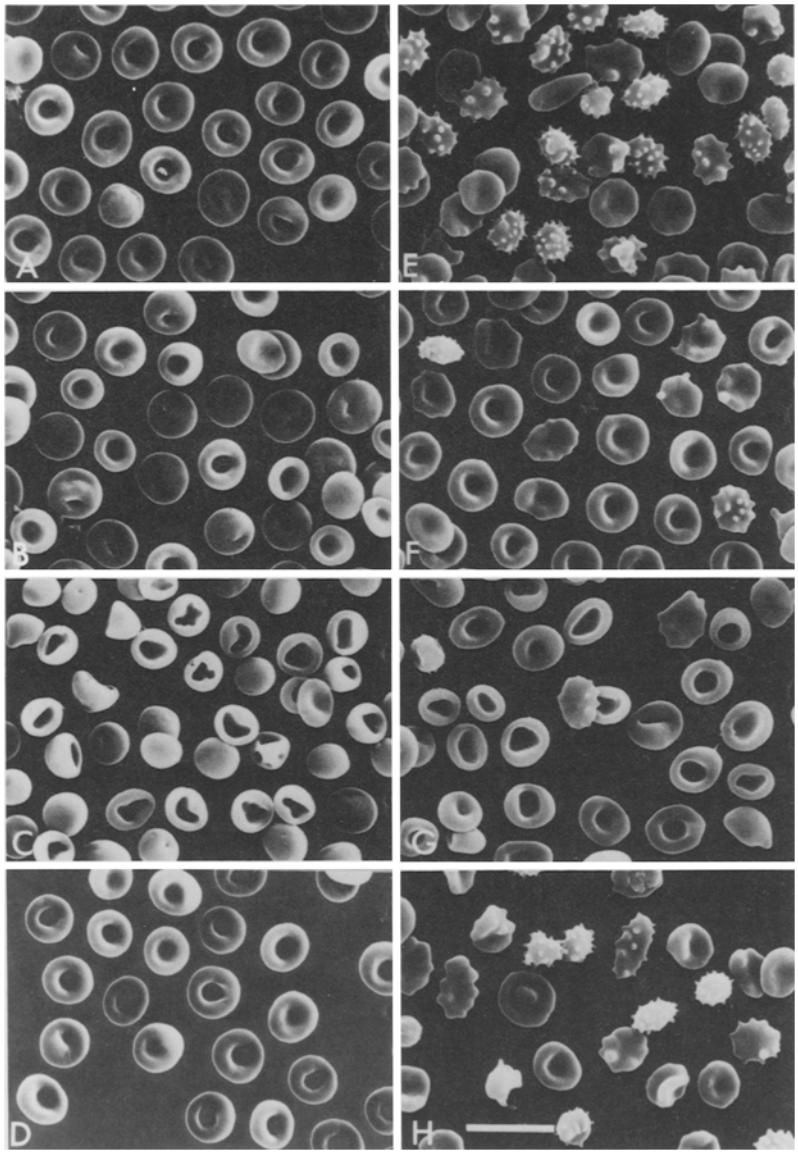

Fig. 3. Effect of chlorpromazine on the contour of echinocytes. The protocol for this experiment was identical to that in Fig. 1. Panels *A–D*, normal cells; panels *E–H*, red cells from a patient with benign echinocytosis. Chlorpromazine (mM): 0 (panels *A* and *E*); 0.03 (panels *B* and *F*); 0.1 (panels *C*, *D*, *G* and *H*). In panels *D* and *H*, the cells were washed thrice in buffer to free them of chlorpromazine prior to fixation. Calibration bar = 10 μm

broad and regular projections (echinocyte-like) were more affected by chlorpromazine than the cells with irregular contour and few projections (acanthocytes). The effects of chlorpromazine on both the normal and diseased red cell were reversed simply by washing away the drug (Fig. 1 *D* and *H*).

Acanthocytes: Isolated Membranes

Whereas ghosts prepared in low ionic strength, alkaline buffers such as 5 mM NaPi (pH 8) from either normal erythrocytes or experimentally induced echinocytes are typically monoconcave disks [14], the ghosts from the patient's blood were as spiculated as the parent cells (Fig. 2 *A vs. E*). While the presence of 0.1 mM chlorpromazine caused only a broadening of the concavity in normal ghosts, it converted the spiculated ghosts to smooth dimpled disks which closely resembled the normal control (Fig. 2 *B vs. F*).

We and others have previously observed that ghosts crenated by treatment with dinitrophenol or isotonic saline revert to a smooth, essentially normal contour in a time- and temperature-dependent fashion (*cf.* ref. 14). We have interpreted this effect as a manifestation of redistribution of lipids so as to normalize the relative area of the two surfaces of the bilayer [14]. An experiment based on this hypothesis was performed on the abnormal ghosts. Whereas warming at low ionic strength for 3 min at 37 °C caused normal ghosts only to be-

come more plump (Fig. 2*C*), it abolished the spicules in ghosts from the patients and induced an essentially normal morphology (Fig. 2*G*). After this warming step, control ghosts were no longer crenated by isotonic saline (Fig. 2*D; see also* ref. 14), while the ghosts from abetalipoproteinemic cells still crenated in 0.15 M NaCl (Fig. 2*H*).

Echinocytes

We examined the erythrocytes of a male donor known to have asymptomatic echinocytosis for many years. The results were qualitatively similar to those described above for the abetalipoproteinemic blood in nearly every respect and will therefore be summarized only briefly. The cells in the fresh, untreated whole blood sample were distorted by many slender projections characteristic of echinocytosis [4]. The morphology was retained by cells washed in saline (Figs. 3*A* and *E*). The presence of 0.03 mM chlorpromazine converted most of these echinocytes to smooth biconcave disks (Figs. 3*B* and *F*). These differed from untreated normal cells (panel *A*) in having shallow dimples and slightly irregular surfaces. Raising the chlorpromazine to 0.1 mM caused deep cupping (stomatocytosis) in the normal and, to a smaller extent, the echinocytic cells (Figs. 3*C* and *G*). The effect of chlorpromazine was entirely reversible upon washing (Figs. 3*D* and *H*).

Unlike abetalipoproteinemic ghosts (Fig. 2), those isolated from the echinocytes in 5 mM NaPi (pH 8) were smooth disks. However, when the ionic strength was raised by the addition of 15 mM NaPi (pH 8), the ghosts from the echinocytes became strongly crenated while their normal counterparts remained smooth monoconcave disks. Ghosts from both normal and abnormal erythrocytes became fully crenated in isotonic saline. As in the case of abetalipoproteinemia, ghosts from echinocytes but not control cells were crenatable in isotonic saline after warming at 37 °C for 3 min at low ionic strength. We infer that the membranes from the echinocytes had a relatively increased potential for evagination.

Discussion

These experiments favor the hypothesis that the mechanism underlying acanthocytosis and echinocytosis *in vivo* is the same as crenation *in vitro;* that is, an excess of surface area in the outer leaflet of the membrane bilayer induces outward curvature by a bilayer couple mechanism [10, 21]. Four types of evidence support this premise:

(1) Where examined carefully, spiculation is induced *in vitro* by amphipaths which accumulate in the outer leaflet of the bilayer either through kinetic trapping (e.g., lysolecithin; ref. 19) or by equilibration between the leaflets [18]. It appears that a deficiency in low density lipoproteins in abetalipoproteinemia causes abnormalites in the serum lipid profile which, through equilibration with the red cell, alter the surface area relationship between the two leaflets of its bilayer. A similar effect apparently operates in the spur cell anemia of severe liver disease, where plasma of abnormal lipid composition can be shown to impose spiculation even on normal red cells *in vitro* [6].

(2) Chlorpromazine, which bends the membrane inward in normal cells [9], reversed spiculation in normal red cells crenated *in vitro* with dinitrophenol [9, 21, 22] and in abnormal red cells (Figs. 1 and 3). That the effect was rapid, immediately reversible, and duplicated in isolated ghosts (Fig. 2) implies that the chlorpromazine does not act through metabolic pathways within the cell but directly on the membrane. We doubt that chlorpromazine acts by binding to calmodulin, as suggested [20], since the submembrane network of human ghosts is almost entirely free of calmodulin [1, 23], yet the ghosts closely resemble intact cells in their response to the drug.

(3) Just as briefly warming normal ghosts in dilute buffer abolishes crenation *in vitro,* so does it smooth the spicules of membranes from abetalipoproteinemic cells (Fig. 2) and from echinocytes. We have suggested that this effect is caused by the flux of excess lipid from the outer to the inner leaflet of the bilayer, perhaps around the perimeter of the holes in the unsealed ghosts [14]. That the ghosts from acanthocytes warmed at low ionic strength were more crenatable than controls suggests that their excess outer surface area had not been completely dissipated.

(4) The networks of spectrin and actin isolated in Triton X-100 from acanthocytes [16] and membranes crenated *in vitro* [15] appear to be smooth and not spiculated. (Our preliminary observation on the networks from the diseased cells described above support this contention [16] but are not definitive.) It seems that spiculation is imposed by the distorted bilayer on the reticulum; once free, the reticulum returns to a smooth contour through elastic recovery [15]. In marked contrast, detergent-extracted membranes from elliptocytes [25] and irreversibly sickled erythrocytes [17] yield correspondingly misshapen reticula; in these cases, the networks appear to maintain the abnormal shape of the cell. [It may be that the small number of

cells which resist the effect of chlorpromazine (e.g., Fig. 1 *F* and *G*) have undergone a secondary alteration in their networks which then preserves their abnormal shape.]

Recently, Alhanaty and Sheetz [2] reported that red cells caused to crenate acutely with dinitrophenol returned to a normal contour during an overnight incubation at 37 °C in a physiologic buffer containing glucose. These authors proposed that the cell senses the contour of the membrane and can correct abnormal shapes. However, it is striking to us that the cells studied here appear to remain distorted in shape throughout most of their life span *in vivo,* yet assume the shape of biconcave disks as soon as the appropriate amphipath is added. We therefore prefer a different hypothesis. We propose that both normal and abnormal red cells are programmed to anticipate a normal lipid profile in their environment. If an abnormal plasma environment supplies an excess of amphipath to the outer surface of the bilayer, stable spur cells, acanthocytes or echinocytes can arise simply because the cells are unable to sense and/or correct the imbalance in area between the two surfaces of the bilayer. That is, there is no apparent mechanism for shape homeostasis *in vivo.*

A distinction is often made between the morphology of the echinocyte and the acanthocyte [4]; the former has many fine spicules evenly distributed over its surface, while the latter has only a few broad spicules of varied length, with rounded ends and an irregular distribution. While these morphological differences may be diagnostically useful, we suggest that the data discussed herein point to a common final pathway for many forms of membrane evagination; i.e. a bilayer couple mechanism. This inference derives from the fact that membranes crenated *in vitro* by dinitrophenol or lysolecithin, acanthocytes from patients with abetalipoproteinemia, and echinocytes all behave similarly in the several tests we have described.

Why, then, is there a difference in spicule morphology between acanthocytes and echinocytes? For example, why is there not one large projection rather than many small ones? We suggest that the answer may lie in lateral phase behavior. That is, each projection may represent a microdomain within the bilayer whose composition is different from the average and favors the local curvature observed. Recent evidence supporting this hypothesis is the demonstration that stearyl alcohol induces only one or two very long projections per red cell; furthermore, the bilayer of the spicules is enriched in the alcohol and is different in its phospholipid profile from the rest of the membrane [12]. How else besides lateral phase inhomogeneity can the bilayer couple hypothesis explain compound curvature in a bilayer? Examples of this situation are the narrow spicules issuing from broad processes in echinoacanthocytes [4] and the coexistence of regions of opposite curvature (spicules and deep cupping) in stomatoacanthocytes [26] and in cells treated with stearyl alcohol [12]. The possibility that the concavities in the normal human erythrocyte reflect lateral (phase) inhomogeneity is an important hypothesis to be tested.

Soon after the first description of the syndrome associated with abetalipoproteinemia [3], Switzer and Eder [24] performed the first "bilayer couple" experiment we know of: they demonstrated that 10^{-4} M Tween 80 and certain other, unspecified detergents restored a normal shape to acanthocytes and experimentally induced echinocytes. They also noted antagonism between agents biasing membrane curvature in the two directions. Weed and Bessis [26] later interpreted the brief report of Switzer and Eder [24] to signify that the intercalated amphipath did not reverse the abnormal curvature of the acanthocyte but merely added cupping, inducing stomatoacanthocytes. While, as noted above, we maintain that both outward and inward curvature can co-exist in the same cell because of lateral inhomogeneity in the bilayer, our data do not support the suggestion of Weed and Bessis [26].

Our results add strength to the hypothesis that spiculation in red cells represents an imbalance in the area of the two membrane surfaces; i.e. a disordered bilayer couple [21] favoring evagination.

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