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# Shape Determinants of McLeod Acanthocytes

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**Summary.** We have sought to elucidate the spiculated shape of McLeod erythrocytes. Red cells from a normal donor and from a McLeod patient were incubated in phosphate-buffered saline containing 0, 0.05, or 0.1 mM chlorpromazine at 0°C for 5 min, then glutaraldehyde-fixed, and examined by scanning electron microscopy. The normal red cells were biconcave disks in which chlorpromazine induced inward (negative) curvature: deep cupping (stomatocytosis) and multiple invaginations. The McLeod cells were mostly spiculated. Chlorpromazine at lower concentration converted them into biconcave disks and, at higher concentration, into stomatocytes. These results support the hypothesis that the spiculation of McLeod cells is the result of an imbalance of surface area between the two lipid leaflets of the membrane; that is, a bilayer couple effect.

We determined the numerical density of intramembrane particles (*IMP*) in replicas of both fracture faces of red cells subjected to freeze fracture and rotary shadowing. These values were as follows (expressed per $\mu m^2$ of membrane ± SD): the normal protoplasmic fracture face had 2200 ± 306 and the McLeod had 2300 ± 250. The normal exoplasmic fracture face had 388 ± 75 and the McLeod had 330 ± 59. We conclude that there is no evidence for derangement of band 3, the principal protein in the *IMP*, in McLeod erythrocytes.

**Key Words** acanthocytes · bilayer · cell shape · chlorpromazine · erythrocytes · lipid · McLeod · membranes · organization

## Introduction

Red cell shape is a classic problem for cell biologists. The induction of shape changes by membrane-intercalated amphipaths has been explained by the bilayer couple hypothesis [30]: increased lipid in the outer leaflet causes evagination or spiculation (echinocytosis), while agents such as chlorpromazine, which expand the inner leaflet, exert an invaginating or stomatocytic effect. However, red cell shape abnormalities have also been ascribed to membrane skeleton proteins [1, 2, 5, 12, 18, 20, 22].

Abnormal shapes are characteristic of certain red cell disorders. These natural systems provide valuable opportunities for investigating the molecular basis of red cell shape. The rare McLeod phenotype [3] is inherited through an X-linked gene and is found only in males [24]. The McLeod red cell is characterized by weak antigenicity in the Kell blood group system, absence of an otherwise ubiquitous antigen named Kx, spiculated morphology (called acanthocytic) and reduced survival in vivo [25, 38]. There is no evidence of gross abnormalities in the membrane proteins [10, 33]. While the composition and distribution of membrane phospholipids in the McLeod red cells are nearly normal, the transbilayer mobility of phosphatidylcholine has been reported to be enhanced [16]. The deranged shape has been attributed to an increased dissociation of band 3 tetramers to dimers *in situ* [11]. Band 3 is the principal integral membrane protein, the major freeze-fracture particle, and the anion transporter in the red cell [13, 35–37]. This correlation of shape abnormality with band 3 was of particular interest to us, since we have found a high density of this integral membrane protein in the thin ellipsoidal cells of the llama [14].

The goal of the present study was to test whether a bilayer couple mechanism or an abnormality in band 3 disposition could account for the spiculated shape of McLeod red cells. A preliminary report of some aspects of these studies has been published [15].

## Materials and Methods

Blood from a normal donor and a patient with the McLeod phenotype was collected by venipuncture in EDTA (10 mM final) and washed in PBS [150 mM NaCl–5 mM $NaP_i$, pH 7.4] at 0°C. Cationized ferritin was obtained from Miles-Yeda Co. All other chemicals were reagent grade or better and were obtained from Fisher.

### Scanning Electron Microscopy

SEM was done on fixed cells that were washed twice in deionized water, spread on glass slides and air dried. Samples were coated with gold-palladium (60:40) and photographed with a JEOL JSM, 3SC scanning electron microscope. The morphology of red cells was interpreted according to Bessis [4].

### Labeling with Ferritin

Labeling with cationized ferritin was applied to a mixed population as follows: normal red cells were briefly fixed in 0.5% glutaraldehyde and labeled with cationized ferritin [8] while McLeod red cells, similarly fixed, were left unlabeled. Each of these samples was additionally fixed separately in 1% glutaraldehyde, washed in PBS and then mixed in equal proportions.

### Freeze-Fracture Electron Microscopy

Red cells of the normal donor and the patient with the McLeod phenotype were fixed in 1% glutaraldehyde and washed in distilled water. The cells were next suspended in 20% glycerin in water and prepared for freeze-fracture electron microscopy as described previously [35]. In addition, a sample of the mixed population was washed in distilled water and prepared for freeze fracture and deep etching. Samples were rapidly quenched in Freon 22 to −150°C. Freeze-fracture and deep-etch procedures were done in a Balzer BAF 301 device by method of Moor and Mühlethaler [26]. For deep etching, the stage temperature was maintained at −100°C for 60–90 sec. Rotary shadowing was at a 25° angle with a Balzer rotary showing unit [23]. Replicas were photographed with a Philips 301 electron microscope.

### Quantitative Electron Microscopy

Electron micrographs of rotary-shadowed replicas of fixed and glycerinated membranes were used at final magnification of 250,000. A 10 × 10 cm transparent grid subdivided into 100 1 × 1 cm squares was superimposed on each micrograph and the particles within that area were counted [14]. Counts were done on 13 cells in each category.

## Results

### Effect of Chlorpromazine on Cell Shape

Normal donor red cells had a biconcave disk shape and only rarely had other forms (Fig. 1). The red cells of the McLeod patient were spiculated (Fig. 4). The early literature named such cells acanthocytes [4, 31]; however, the mechanistic basis for this terminology is unclear [32]. Various stages of acanthocytes were identified according to criteria established for identification of different stages of echinocytes [4]. The cells of our patient were 34% acanthocytes of Stage I (irregularly contoured disks) and 32% Stage II and Stage III (flat cells with spicules and ovoid or spherical cells with 10–30 spicules, respectively). There were no Stage IV acanthocytes (spherical cells with needle-like projections). In all, 66% of the McLeod red cells were spiculated, a value similar to that discussed in the literature [27].

Chlorpromazine induced a dose-dependent shape change in the normal red cells. At 0.05 mM, it caused the conversion of over 25% of the biconcave disks to stomatocytes (Fig. 2). At 0.1 mM, chlorpromazine converted 70% of the biconcave disks to stomatocytes, some of which had numerous microinvaginations (Fig. 3).

In the presence of 0.05 mM chlorpromazine, approximately 70% of the McLeod red cells were converted to the biconcave disk form and 10% to the stomatocytic form (Fig. 5). In the presence of 0.1 mM chlorpromazine, the shape of the McLeod red cells became 51% stomatocytic and 37% biconcave (Fig. 6). Microinvaginations were rare in these cells. Thus, chlorpromazine converted normal biconcave red cells into stomatocytes and acanthocytes into biconcave disks.

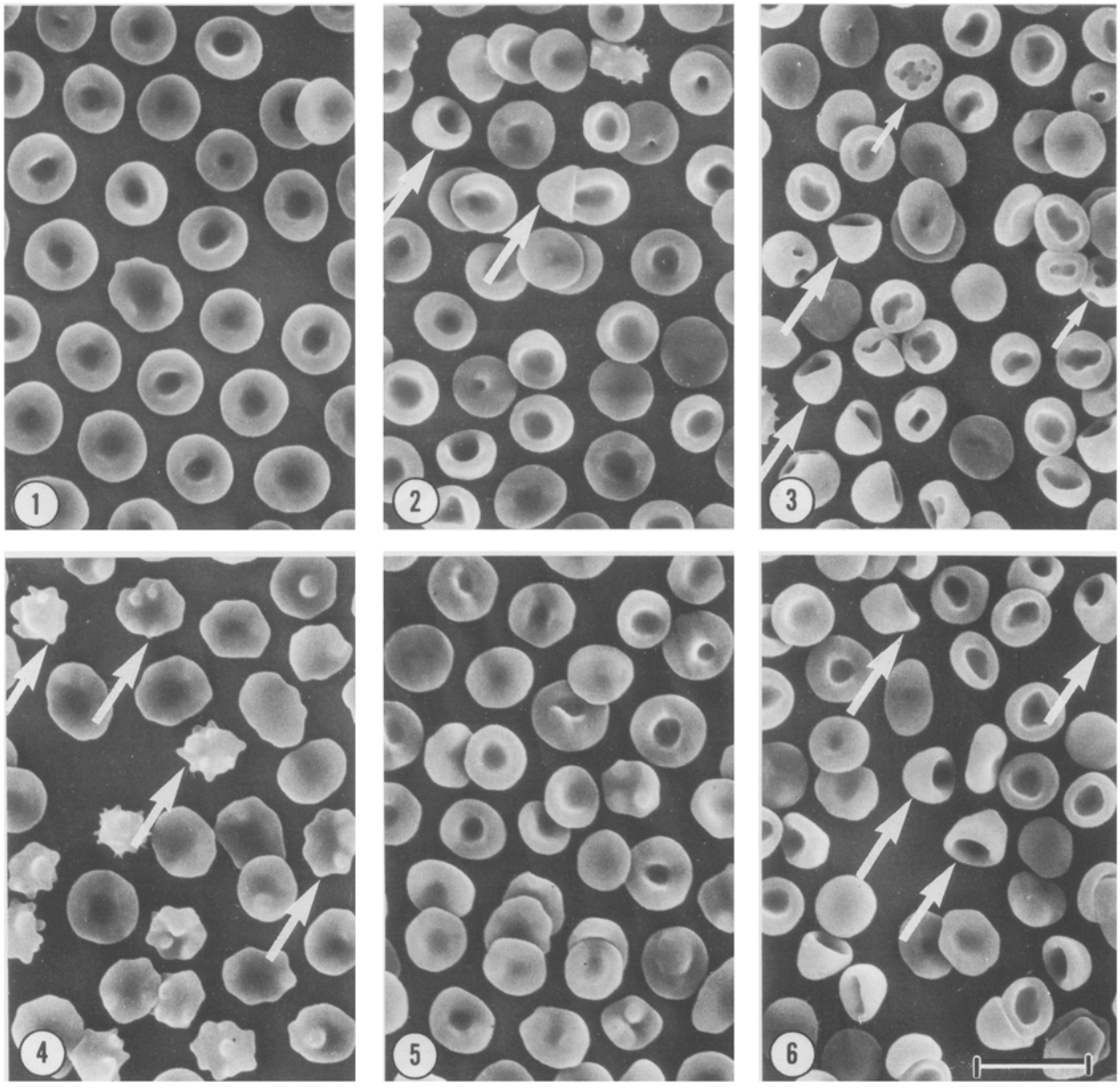
### Intramembrane Particles (IMP)

The distribution of *IMP* was assessed on replicas of freeze-fractured and deep-etched membranes. In order to make an internally controlled comparison of normal and McLeod red cells, we examined 1 : 1 mixtures of the two, having first labeled the former with ferritin for identification purposes. The numbers of *IMP* on the protoplasmic fracture faces appeared similar in normal (Fig. 7) and McLeod red cells (Fig. 8).

Quantitative electron microscopy of *IMP* was done on separate replicas of rotary-shadowed, freeze-fractured membranes of glutaraldehyde-fixed and glycerinated red cells. The sizes of *IMP* appeared similar on the protoplasmic fracture faces of normal (Fig. 9) and McLeod red cells (Fig. 10) as well as on their extracellular fracture faces (Figs. 11 and 12), and the numerical densities were not significantly different ($P < 0.01$). The numerical densities of the *IMP* are given in the Table.

## Discussion

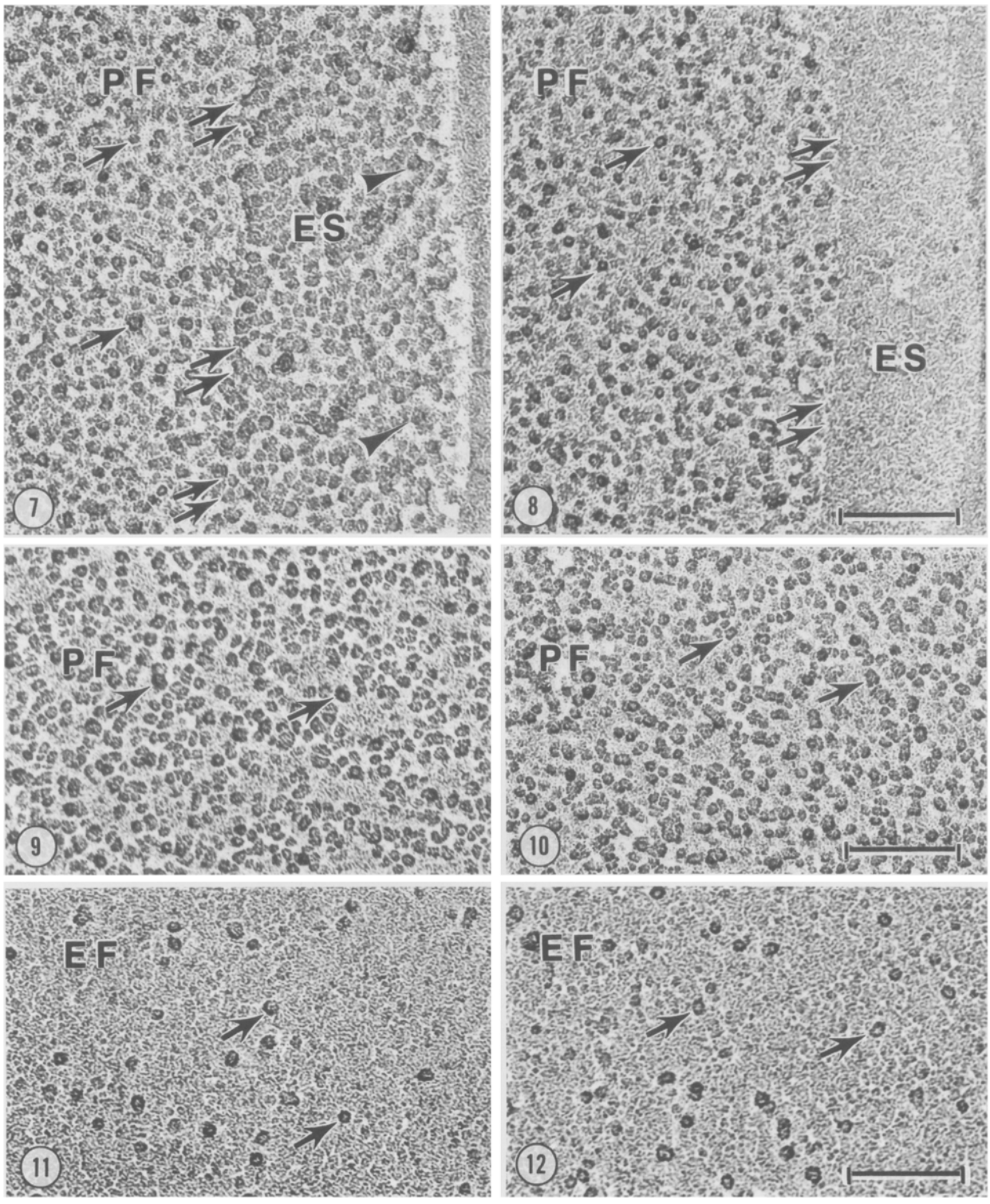
Our data show that the numerical density of *IMP* in normal and McLeod red cells is similar (see Table). In contrast, Glaubensklee et al. [11] reported an increase in the numerical density and a decrease in particle size of *IMP* in McLeod red cells. They inferred that the normal tetramers of band 3 dissociate to dimers in the McLeod membranes and that




**Figs. 1–6.** Effect of chlorpromazine on the shape of red blood cells. One volume of packed red cells washed in PBS was incubated at 0°C for 5 min in 8 volumes of PBS (pH 7.0) containing chlorpromazine. Samples were then fixed with 1% glutaraldehyde for scanning electron microscopy. Figures 1–3 are of normal cells and Figs. 4–6 of McLeod cells treated with 0, 0.05, and 0.1 mM chlorpromazine, respectively. Large arrows point to stomatocytes (Figs. 2, 3 and 6) and spiculated cells (Fig. 4). Small arrows point to red cells with microinvaginations. Bar = 10 $\mu$m

this caused their spiculated shape. The discrepancy between the results of Glaubensklee et al. [11] and ours might be explained by differences in the platinum/carbon shadowing of freeze-fractured faces of membranes. Our studies were based on replicas of rotary-shadowed membranes, while Glaubensklee and coworkers [11] used unidirectionally-shadowed material. Rotary shadowing eliminates certain ambiguities in particle identification and therefore has advantages over unidirectional shadowing for determination of numerical densities of *IMP* [23, 34]. Since the *IMP* appear normal in size and number in the McLeod cells, there is no reason to assign them a role in the shape abnormality. This conclusion is supported by the data of Redman et al. [29].

Despite some controversy [7], the action of chlorpromazine on the red cell has been shown to result from its membrane intercalation [6, 9, 19, 21,




**Figs. 7 and 8.** Replicas of a deep-etched and rotary-shadowed red cell membrane from the normal (Fig. 7) and the McLeod donor (Fig. 8). Both cells were present in the same replica and were distinguished by ferritin label affixed to the outer surface of the normal cells (*see* text). The following designations are used: *ES* (extracellular surface) and *PF* (protoplasmic fracture face). Arrowheads point to ferritin particles. Double arrows show where the fracture passes from the interior of the membrane to the extracellular surface. Single arrows point to *IMP*. Bar = 0.1 $\mu$m

**Figs. 9–12.** Replicas of rotary-shadowed fracture faces of glutaraldehyde-fixed and glycerinated normal (Figs. 9 and 11) and McLeod (Figs. 10 and 12) red cells. *PF* designates the protoplasmic fracture face and *EF* designates the extracellular fracture face. Arrows point to *IMP*. Bar = 0.1 $\mu$m

**Table.** Numerical density of intramembrane particles in red cell membranes

| | Normal | McLeod |
|---|---|---|
| | (Particles per $\mu m^2$) | |
| Protoplasmic fracture face | 2200 ± 306 SD[a] | 2300 ± 250 SD |
| Extracellular fracture face | 388 ± 75 SD[a] | 330 ± 59 SD |

[a] As determined by the Student's *t* test, *IMP* numerical densities for normal and McLeod membranes were not significantly different ($P < 0.01$).

28]. The response of McLeod red cells to chlorpromazine suggests that the spiculation is caused by a bilayer couple mechanism. Unlike abetalipoproteinemia [17], the spiculation of McLeod red cells is intrinsic and independent of the plasma [10, 38]. A bilayer couple mechanism is also supported by the finding of enhanced transbilayer mobility of phosphatidylcholine in McLeod red cells [16]. An imbalance of less than 1% between the area of the two leaflets is all that is required to produce the deformations observed [32].

Our study suggests that there is a derangement in the organization of the bilayer in the McLeod red cells. The basis for this bilayer imbalance, however, remains unknown.

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