Membrane Microfilaments of Erythrocytes: Alteration in Intact Cells Reproduces the Hereditary Spherocytosis Syndrome

(vinblastine/colchicine/strychnine/electron microscopy/cell rigidity)

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ABSTRACT Membrane microfilaments are found throughout the animal world in situations suggesting that they fulfill a critical role in providing normal cell shape and plasticity. We have hypothesized that hereditary spherocytosis, a congenital hemolytic anemia associated with intrinsically rigid and mishapen erythrocytes, might result from genetically defective microfilaments in erythrocyte membranes. By using three different drugs (vinblastine, colchicine, and strychnine) that share one common attribute—that of potently precipitating purified microfilamentous protein—we have provided support for this hypothesis. Thus, all the known in vitro and in vivo characteristics of hereditary spherocytes are reproduced in normal erythrocytes briefly exposed to these precipitating agents.

Hereditary spherocytosis, the most prevalent congenital hemolytic anemia of man, affects roughly 0.02% of all humans. Evidence has accumulated that an intrinsic defect in the erythrocyte membrane underlies this disease. This evidence includes: (a) erythrocyte membranes in this disease leak sodium at excessive rates (1, 2); (b) “buds” of membrane form prematurely, and fragment off the hereditary spherocyte during incubation, thereby enhancing spherocytosis (3, 4); (c) erythrocyte ghosts from affected individuals are intrinsically stiffer than normal, resisting passage into glass microcapillary tubes (5); and (d) these membranes have a unique “wrinkled” appearance when they are viewed with surface-scanning electron microscopy (6). The nature of the membrane defect has recently been studied through examination of proteins extracted from erythrocyte ghosts. In contrast to proteins from normal erythrocyte membranes, those extracted at low ionic strength from membranes of patients with spherocytosis resist aggregation when the ionic strength is increased (7). Others have shown that microfilaments with ultrastructural characteristics similar to actin are generated from proteins purified from erythrocyte membranes when ionic strength is increased with divalent cations (8). Based on these observations, we have suggested that defective formation of such microfilaments might explain the increased rigidity and, thereby, the diminished survival of the hereditary spherocyte (7). Support for this supposition comes from accumulated evidence that the shape and motility (or “contractility”) of cells derive from structural elements such as microtubules and microfilaments (9). The molecular characteristics of these morphologic elements allow a useful hypothesis: that is, structural proteins are a class of similar, but not identical, molecules (10). Furthermore, there are indications that the microfilament proteins that may underlie cell shape share important properties—for example, binding of myosin and precipitation by alkaloids such as vinblastine, colchicine, and strychnine—with molecules responsible for motility, such as actin filaments of muscle and the microfilaments involved in cytoplasmic streaming (11, 12). Our recent observation (7) that protein extracted from hereditary spherocyte membranes precipitates less readily than does normal membrane protein when it is treated with vinblastine supports the possibility that defective membrane microfilaments might lead to the intrinsic rigidity and peculiar shape of the hereditary spherocyte. This paper provides further evidence to support this concept.

We have used the rather specific capability of three diverse compounds (vinblastine, colchicine, and strychnine) to precipitate microfilament proteins to provide further proof that hereditary spherocytosis reflects an inborn defect in this class of proteins. All three compounds reversibly precipitate microfilament proteins extracted from such disparate sources as cilia, mitotic spindles, erythrocyte membranes, and skeletal muscle (actin) (13). In all situations, precipitation is reversed by removal of the drug by dialysis. Our studies indicate that brief exposure of normal erythrocytes to any of the three compounds generates cells identical to the hereditary spherocyte.

MATERIAL AND METHODS

Heparinized blood was obtained from normal volunteers and three unrelated spherocytic donors (two of whom were splenectomized). Erythrocytes were washed three times in phosphate-buffered isotonic saline (pH 7.4; [PO₄] = 12 mM; [glucose] = 10 mM) before use. Vinblastine, colchicine, and strychnine* were dissolved in isotonic saline and added at the appropriate concentration to a 30% suspension of erythrocytes in phosphate-buffered saline. Unless otherwise stated, the final concentration of vinblastine was 0.2 mM; completely analogous results were obtained with roughly 10-times higher concentrations of colchicine and strychnine. The loss of erythrocyte membrane through “budding” was quantitated by analyses of total membrane lipid (mainly phospholipid and cholesterol) during incubation (3, 4). Erythrocyte rigidity was estimated by the resistance of the cells to filtration by a modification of the paper-filtration

* Vinblastine sulfate was obtained from Eli Lilly Co., Indianapolis, Ind.; colchicine and strychnine were obtained from Sigma Chemical Co., St. Louis, Mo.
Prolonged incubation significantly (left); hemolysis is shown (15). The filter paper was supported in a glass funnel, which was used throughout the studies. The rate of filtration was measured by continuously collecting the filtrate in a tared vessel placed on a Mettler balance pan. Weights of filtrate at 30-sec intervals were plotted on semi-logarithmic paper to give a straight line. The rate of filtration is directly proportional to erythrocyte plasticity (i.e., inversely proportional to erythrocyte rigidity). Erythrocyte survival and organ sequestration studies of cells exposed to drugs were performed with Na$_2$CrO$_4$-labeled erythrocytes as described (15). Unreacted vinblastine, colchicine, or strychnine was removed by a rapid wash in a 100-fold excess of saline just before injection of the cells.

The ultrastructure of drug-treated erythrocytes was examined by scanning electron microscopy with a Cambridge stereoscan electron microscope, after preparation of the cells in 1% glutaraldehyde. Transmission electron micrographs were prepared (16); thorium dioxide was used as an electron-dense surface marker.

RESULTS

Brief exposure to vinblastine, colchicine, or strychnine reproduces in normal erythrocytes all the known in vivo and in vitro characteristics of hereditary spherocytes. Results with all three drugs were identical, except that 10-times more colchicine and strychnine were required than vinblastine. To conserve space, we present only the results with vinblastine here.

Normal erythrocytes exposed to vinblastine for 1 hr become morphologically identical to hereditary spherocytes (right, Fig. 1). Microspherocytosis occurs without volume change (as measured by hematocrit) or external membrane loss (as measured by lipid content). These cells are of increased osmotic fragility (left, Fig. 2); the fragility curves are characteristic of those observed in the genetic disease. Although the effect is not detected in short-term experiments, after overnight incubation as much as 20% of the membrane lipid is lost from treated erythrocytes into the medium (right, Fig. 2). As in spherocytosis, the lipid moieties (phosphatides and cholesterol) are lost in exact proportion to their content in the fresh cell. Thus, “buds” of membrane are lost during prolonged incubation of treated cells exactly as observed in hereditary spherocytosis (3, 4).

Another of the characteristics of hereditary spherocytes is their increased permeability to sodium. A concomitant increase in active pumping of the cation, with an appropriately increased glycolytic rate, follows (1). These abnormalities are reproduced in normal erythrocytes that are briefly exposed to vinblastine, colchicine, or strychnine. Influx and efflux rates (measured with $^{22}$Na) are roughly doubled in

**TABLE 1. Reversible induction of rigidity in vinblastine-treated erythrocytes**

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Filtration half-time</th>
<th>Rigidity (times normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range (10)</td>
<td>1.2-2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Hereditary spherocytes (3)</td>
<td>4.0-7.8</td>
<td>2.5-4.9</td>
</tr>
<tr>
<td>Vinblastine-treated (3)</td>
<td>3.2-4.0</td>
<td>2.0-2.5</td>
</tr>
<tr>
<td>after dialysis (3)</td>
<td>1.0-2.0</td>
<td>0.6-1.3</td>
</tr>
</tbody>
</table>

* The rates of filtration through moistened filter papers of washed erythrocyte suspensions (50%) from normal volunteers and three patients are compared. Erythrocytes treated for 1 hr at 37°C with 0.2 mM vinblastine were similarly filtered in three experiments before and after 4 hr of drug-removal by dialysis. “Rigidity” is estimated by comparison of filtration half-times with those of normal erythrocytes.
treated erythrocytes; concomitantly, glycolytic rates increase 20%. This increase is prevented if active pumping of sodium is inhibited by ouabain.

The rigidity of hereditary spherocytes is increased; the half-time of their filtration through paper filters is roughly 3 to 5 times that of normal erythrocytes (Table 1). Exposure of normal erythrocytes to vinblastine (or colchicine or strychnine) for 30 min produces increases in erythrocyte rigidity (decreases in filtration rate) comparable to those observed with hereditary spherocytes. After removal of the drug by dialysis for 3 hr, the erythrocytes regain normal plasticity, as shown by normalization of their filtration rates (Table 1).

The most unique characteristic of hereditary spherocytes in vivo is their specific entrapment and destruction in the spleen. When injected into normal subjects, vinblastine-treated erythrocytes labeled with 51Cr behave identically (Fig. 3). Rapid removal of labeled erythrocytes (upper left, Fig. 3) is accompanied by specific uptake of the label by the spleen (lower left, Fig. 3). However, as with hereditary spherocytes, these cells survive normally when injected into a splenectomized patient, and no evidence of hepatic sequestration is found (right, Fig. 3).

The fact that the rapid change in shape, and concomitant increase in osmotic fragility, of vinblastine-treated erythrocytes (Figs. 1 and 2) occur without volume change suggests that these cells lose membrane. However, at times when spherocytosis is well-marked, no external loss is demonstrable by assay of lipid content of the treated cells. This seeming paradox is solved by ultrastructure studies. Scanning electron microscopy of vinblastine-treated erythrocytes demonstrates the emergence of numerous cup- and bowl-shaped cells after about 30 min of exposure (left, Fig. 4). These shapes suggest that invagination of membrane is occurring by a "purse-string" type of contractile process. When opposing edges of uninvaginated membrane are resealed, more perfect microspherocytes are fashioned, some still containing dimples where invaginations originally occurred (left, Fig. 4). Sections of treated erythrocytes viewed by transmission electron micros-

![Image](image1.png)

**Fig. 4.** Scanning and transmission electron micrographs of vinblastine-treated erythrocytes. *Left:* Note bowl- and cup-shaped forms, especially along right border of this scanning electron micrograph; this suggests membrane invagination by a "purse-string" effect. More perfect spherocytes, some with dimples, are noted in the center of the micrograph. *Right:* The external surface of the erythrocyte is marked by electron-opaque thorium dioxide (small black dots). Note membrane invagination and endocytotic vacuoles containing the opaque marker. The amorphous black clump on the left border of the invagination is an artifact.

![Image](image2.png)

**Fig. 3.** Specific splenic destruction of vinblastine-treated erythrocytes. Human erythrocytes treated for 1 hr with 0.2 mM vinblastine and labeled with 51Cr disappear rapidly when injected into the normal circulation (left upper) and specifically accumulate in the spleen (left lower). In a splenectomized subject (right), these same cells survive normally and without hepatic sequestration (lower) — , spleen; O—O, liver.

**DISCUSSION**

These studies expand to human erythrocytes the concept that membrane microfilament proteins are crucial to normal cellular shape and plasticity. Furthermore, the observation that chemical modification of this class of proteins reproduces all the known characteristics of hereditary spherocytes allows the reasonable conclusion that hereditary spherocytosis re-
results from mutations in microfilament proteins. The three alkaloids used in these studies, although significantly different in molecular structure, share the ability to reversibly precipitate various microfilament proteins purified from diverse types of cells (13). The effect of all three drugs is potentiated by calcium, protein precipitation being markedly enhanced in the presence of this cation (13). It is of interest, therefore, that Ca++ has recently been demonstrated to produce rigid spherocytes from normal erythrocytes (or their ghosts) when it accumulates intracellularly (17). As with the alkaloid drugs used in the present studies, this effect of Ca++ is reversed by removal of the perturbing cation. We suggest that calcium and the alkaloid drugs used herein act on identical microfilament proteins in the erythrocyte membrane to reversibly alter their conformation in such a way as to result in cell rigidity.

We also note a further identity between the characteristic properties of the alkaloids to precipitate purified microfilament proteins and their capacity to induce spherocytosis of intact erythrocytes. That is, 10-times more colchicine and strychnine are required than vinblastine both to precipitate purified microfilamentous proteins (e.g., actin) (13) and to generate spherocytes. Furthermore, the solubilization by dialysis of alkaloid-precipitated, purified protein finds analogy with the reversal of rigidity in vinblastine-treated erythrocytes after removal of the drug by dialysis (Table 1). The reversibility of the rigidification process probably explains our observations that only some vinblastine-treated erythrocytes are rapidly removed from the circulation by the spleen; cells that survive for 8 hr or longer then survive perfectly normally without evidence of further splenic entrapment. We presume that the drug effect has been "washed-away" during circulation for several hours in a drug-free environment.

Whether a single molecular species of microfilament protein, or several such proteins, cause normal erythrocyte shape and plasticity is not known. However, we do have preliminary evidence (18, 19) that different mutations in membrane protein are found in different families with the spherocytosis syndrome. It is our view that any mutation that inhibits the aggregation into microfilaments of protein subunits of this class might result in a rigid, and thereby, poorly viable erythrocyte. This view is in concert with the variable severity of the disease in different families.

These results have provoked us to examine the effect of the alkaloid drugs on other intact cell membranes. Vinblastine is commonly used as a chemotherapeutic agent for various malignancies, especially the leukemias. The amount of this drug required to stiffen erythrocytes is more than 10-times higher than that probably present in the treated patient. Nonetheless, it will be of interest to assess the rigidity and thus the motility of cells such as granulocytes in vinblastine-treated patients. For example, it would seem especially disadvantageous for patients with leukemia and, consequently, diminished numbers of normal granulocytes, to have the mobility of these cells inhibited (e.g., into areas of bacterial growth).

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