Marginal bands (MBs) of microtubules (MTs) are believed to function during morphogenesis of nonmammalian vertebrate erythrocytes, but there has been little evidence favoring a continuing role in mature cells. To test MB function, we prepared dogfish erythrocytes with and without MBs at the same temperature by (a) stabilization of the normally cold-labile MB at 0°C by taxol, and (b) inhibition of MB reassembly at room temperature by nocodazole or colchicine. We then compared the responses of these cells to mechanical stress by fluxing them through capillary tubes. Before fluxing, cells with or without MBs had normal flattened elliptical shape. After fluxing, deformation was consistently observed in a much greater percentage of cells lacking MBs. The difference in percent deformation between the two cell types was highly significant. That the MB is an effector of cell shape was further documented in studies of the formation of singly or doubly pointed dogfish erythrocytes that appear during long-term incubation of normal cells at room temperature. On-slide perfusion experiments revealed that the pointed cells contain MBs of corresponding pointed morphology. Incubation of cells with and without MBs showed that they become pointed only when they contain MBs, indicating that the MB acts as a flexible frame which can deform and support the cell surface from within. To test this idea further, cells with and without MBs were exposed to hyperosmotic conditions. Many of the cells without MBs collapsed and shrunken, whereas those with MBs did not. The results support the view that the MB has a continuing function in mature erythrocytes, resisting deformation and/or rapidly returning deformed cells to an efficient equilibrium shape in the circulation.

All nonmammalian vertebrate erythrocytes contain a marginal band (MB) of microtubules (MTs) as a prominent cytoskeletal component. The MB is believed to function universally during generation of the characteristically flattened, elliptical shape of such erythrocytes, but whether it has a continuing role in cell shape maintenance is controversial. The correlation between MB thickness (MT number) and red cell size in diverse species (9, 16), as well as apparent MB flexibility (15, 20), have been cited as evidence favoring a maintenance role. However, the decrease in MT number with red cell maturation (1, 25) and, in particular, the maintenance of normal erythrocyte shape when the MB of certain species is disassembled at 0°C (1, 4) have been presented and more widely accepted as evidence against such a role.

Abbreviations used in this paper: MB, marginal band; MT, microtubule; SAC, cell surface-associated cytoskeleton.

Proper examination of MB function in mature erythrocytes requires the study of living cells with and without MBs under otherwise identical conditions, comparing their responses to dynamic treatment. The dogfish erythrocyte can be manipulated to provide a useful system for such studies. The MB of these cells is cold labile, disassembling at 0°C and reassembling upon rewarming at room temperature (12). Using MT-inhibiting or -stabilizing drugs, both MB disassembly at 0°C and MB reassembly at room temperature can be blocked, thus generating erythrocytes with and without MBs.

In this paper we report effects of mechanical stress and hyperosmotic conditions on dogfish erythrocytes with and without MBs. We also describe experiments testing the causal relationship between MB shape and cell shape. The results strongly support the contention that MBs have a continuing function in mature erythrocytes and indicate a mechanism by which this function is achieved.
MATERIALS AND METHODS

Chemicals: Taxol (a gift of Dr. M. Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute) and nocodazole (Sigma Chemical Co., St. Louis, MO) were prepared in dimethyl sulfoxide as 5 mg/ml and 2 mg/ml stocks, respectively, and stored frozen. Colchicine (Sigma Chemical Co.) was prepared as a 0.1 M stock and stored frozen.

Collection and Washing of Cells: Freshly drawn blood (= 1 vol) from the smooth dogfish, Mustelus canis, was immediately diluted with 1 vol heparinized (100 U/ml) elasmobranch Ringer’s, which contained 0.1% dextrose as an energy source (7). The cells were collected by centrifugation (2 min at ~1,350 g), the white layer was resuspended and discarded, and the remaining cells were resuspended to 2 vol with heparinized (50 U/ml) Ringer’s. The suspension was layered over 0.5 M sucrose in 25% Ringer’s and sedimented. The pellet, containing mostly erythrocytes, was resuspended and washed several times in elasmobranch Ringer’s, carefully removing any remaining leukocytes by gentle swirling. The packed cells were diluted 1:10 (vol/vol) with elasmobranch Ringer’s to produce the working cell suspension. The hematocrit of this suspension (10%) is approximately one-third that of whole dogfish blood; the latter contains ~500,000 red cells/mm³ as determined by hemacytometer counts.

Generation of Cells with and without Marginal Bands: Cells containing MBs at 0°C were produced by addition of taxol (23) to 10 μg/ml and incubation for 3 h at 0°C. Control cell suspensions lacking taxol but containing an equivalent concentration of dimethyl sulfoxide were similarly incubated, yielding cells without MBs at 0°C.

Cells containing or lacking MBs at room temperature were produced as follows: suspensions of erythrocytes were incubated either with 0.1 mM colchicine or with 10 μg/ml nocodazole (13) for 1 h at room temperature and then placed at 0°C for at least 1 h. By this time all MBs had disassembled. These suspensions, still containing the inhibitor, were then incubated for at least 1 h at room temperature (23°C), generating erythrocytes without MBs. Control cell suspensions containing no inhibitors were temperature-cycled at the same time, producing erythrocytes with reassembled MBs. Dimethyl sulfoxide was included in control suspensions for nocodazole experiments at a concentration equal to that in the experimental ones.

Preparation of Cytoskeletons: To determine whether erythrocytes contained MBs after the drug treatments, cells were lysed under MT-stabilizing conditions. A sample of cell suspension (0.05 ml) was diluted ~1:10 into lysing medium consisting of 100 mMPIPES, 1 mM MgCl₂, 5 mM EGTA, 10 mM p-tosyl arginine methyl ester, and 0.4% Triton X-100 at pH 6.8. This procedure immediately clears hemoglobin from the cells, producing nucleated cytoskeletons in which MBs are readily visible by phase-contrast microscopy (12).

Mechanical Stress Experiments: 10-μl samples of cell suspensions containing erythrocytes with or without MBs were fluxed 10 times at room temperature through 10-μl capillary tubes (400 μm diam; Dade Diagnostics, Inc., Aguada, PR). The flow rate was ~0.5 ml/s on efflux. The 10-μl suspensions were then immediately fixed in 2% glutaraldehyde in elasmobranch Ringer’s and viewed by phase-contrast microscopy. Nonelected fields were counted, scoring a minimum of 200 cells for deformed vs. normal shape. Some samples were not fixed but immediately viewed by phase-contrast microscopy, verifying that cell shape was not affected by fixation.

Generation of Pointed Cells: Suspensions of erythrocytes with and without MBs were incubated in elasmobranch Ringer’s at room temperature for at least 19 h. Samples were fixed in 2% glutaraldehyde in elasmobranch Ringer’s and viewed by phase-contrast microscopy. Nonelected fields were counted and a minimum of 200 cells were scored for the presence or absence of single or double points.

Perfusion Experiments: Coverslips were coated with Aclarian Blue by allowing a film of the dilute aqueous dye to dry (18). A sample of erythrocyte suspension in Ringer’s was incubated on the coverslip for 2 min to allow cells to adhere. The coverslip was then rinsed briefly with Ringer’s and inverted onto the same medium on a slide, elevated on coverslip fragments. Perfusion was accomplished by placing strips of paper wipes in contact with one another edge and drops of lysing medium along the other. Perfusion rate was controlled by moving the paper strips. Cells were photographed before and after perfusion with lysing medium.

Hyperosmotic Treatment of Cells: Cell suspensions containing erythrocytes with and without MBs were treated with hyperosmotic sucrose solutions. The osmolality of dogfish serum (960 mosmol/kg of H₂O) and of the sucrose solutions was measured with a vapor pressure osmometer (model 5100C; Wescor, Inc., Logan, UT). 1.5 ml of either 1.5 M or 1 M sucrose (1,928 and 5100 mosmol/kg, respectively) was added to 0.5 ml of cell suspensions, and the cells were incubated in this medium for 5 min. Cells were then fixed by addition of 2 ml of 2% glutaraldehyde in the appropriate sucrose solution (1.5 M or 1 M). A minimum of 200 fixed cells in nonelected fields were examined under phase contrast and scored for normal vs. shriveled appearance.

Scanning Electron Microscopy: Cells with and without MBs were fixed in 1% glutaraldehyde in elasmobranch Ringer’s for 1 h, placed on polylysine-coated glass coverslips (0.1% polylysine) and incubated for 10 min. Coverslips were then cleaned through alcohol dehydration (10–100%), critical point drying (Tousimis Research Corp., Rockville, MD) and coating with gold/palladium (Tousimis SAMPPUTTER). Samples were viewed with the JEOL J300 scanning electron microscope (JEOL USA, Electron Optics Div., Peabody, MA) (20 kV).

RESULTS

Production of Cells with and without MBs

The procedures developed to generate cells with and without MBs at the same temperature were based upon previous observations on dogfish and “blood clam” erythrocytes (12, 19). MBs disassembled at 0°C and reassembled upon rewarminig, as shown previously in thin sections and whole mounts (12). The cell population displayed MBs of varying thickness after 1–3 h of rearming, including some that were very thin. When taxol was present, MB disassembly at 0°C was inhibited in all cells (Fig. 1), and either colchicine or nocodazole inhibited MB reassembly at room temperature in all cells. Normal flattened, elliptical morphology was retained by nearly all cells, regardless of whether they contained or lacked MBs (Fig. 1).

Effect of Mechanical Stress on Erythrocytes with and without MBs

The availability of populations of dogfish erythrocytes with and without MBs under similar conditions allowed us to study MB function. To test whether erythrocytes with MBs react to a given mechanical stress differently from those without MBs, erythrocytes were fluxed in capillary tubes. Nearly all of those with MBs remained normal in appearance.
However, many cells without MBs underwent a striking change in morphology, appearing buckled or folded (Figs. 2b and d and 3b). As shown in Table I, in all experiments there was a highly significant increase in the number of deformed cells when MBs were absent.

Cell deformation was attributable to the fluxing itself. Prior to fluxing, cell morphology was normal except for a few cells typically seen in suspensions without MBs, presumably produced by the stress of initial Pasteur pipetting. Cell deformation was not dependent on subsequent fixation, as determined by direct observation of living cells.

**Generation of Pointed Erythrocytes**

When washed dogfish erythrocytes are incubated in elasmobranch Ringer's at room temperature, singly or doubly pointed cells appear, increasing in number with time. Significant numbers of such cells are first observed after ~4 h, with doubly pointed cells accumulating only upon longer incubation. The percentage of pointed cells in a suspension is correlated with the percentage of singly or doubly pointed MBs observed in cytoskeletons prepared from the population (12). An example of such a cytoskeleton is shown in Fig. 4. To verify that singly and doubly pointed cells contain correspondingly pointed MBs, perfusion experiments were performed in which the same cell was viewed by phase-contrast microscopy before and after lysis. As shown in Fig. 5, pointed cells do contain pointed MBs, whereas cells of normal shape contain smoothly curved, normal MBs.

Are MBs necessary for the formation of pointed erythrocytes? Cells with and without MBs at room temperature were
produced as previously described. These cells were then further incubated at room temperature for at least 19 h in the absence or presence of inhibitors, respectively. Even though we counted the most minimal protrusion as a "point" in the samples containing cells without MBs, <5% of these cells had points whereas 27–56% of cells with MBs had points (Table II). Both singly pointed teardrop-shaped cells and doubly pointed spindle-shaped cells were present. For simplicity, the data are reported here as the total number of pointed cells; however, the same result is obtained if one considers the total number of points.

**Effect of Hyperosmotic Treatment on Cells with and without MBs**

Erythrocytes with and without MBs were studied to determine whether MBs maintain cell shape during loss of cell volume. Cells were suspended in sucrose-containing solutions hyperosmotic to dogfish serum. In 1.1 M sucrose–25% (vol/vol) Ringer's, as many as 20% of the cells lacking MBs were shriveled as compared with <1% of cells containing MBs (Fig. 7).
Correlation between MB morphology and erythrocyte morphology. Pointed erythrocytes adhering to coverslip were perfused with LyM, revealing that they contain correspondingly pointed MBs. (a) One cell of normal shape and one doubly pointed cell (arrowheads) prior to perfusion. (b) Cytoskeletons of cells shown in a; normal cell has smooth, continuous MB, whereas doubly pointed cell has a doubly pointed MB (arrowheads). (c) A singly pointed cell containing (d) a singly pointed MB (arrowheads); phase-contrast microscopy. × 1,030.

### TABLE II

**Generation of Points in Cells with and without MBs**

| Experiment | Treatment   | Presence of MB | Pointed cells |
|------------|-------------|----------------|---------------|
| 1          | + colchicine | –              | 1             |
|            | – colchicine | +              | 39            |
| 2          | + colchicine | –              | 4             |
|            | – colchicine | +              | 27            |
| 3          | + nocodazole | –              | 1             |
|            | – nocodazole | +              | 56            |
| 4          | + nocodazole | –              | 4             |
|            | – nocodazole | +              | 39            |

Cells with and without MBs were incubated at room temperature for 19-22 h. A minimum of 200 cells per experimental treatment was scored for the presence or absence of points.

The result was similar in 0.75 M sucrose–25% (vol/vol) Ringer’s, in which as many as 12% of the cells lacking MBs were shrunken (Table III).

Because mechanical stress has a profound effect on cells without MBs (Figs. 2 and 3), care was taken in the design of this experiment to minimize mechanical stress. Centrifugation was avoided. Cells were pipetted initially, when they all contained MBs, but were not pipetted again until after fixation.

During the experiment, mixing of cells with the sucrose solution was done by gentle inversion. Such mixing had a minimal mechanical effect as shown by a control experiment using 100% Ringer’s. In addition, the typical appearance of affected cells in hyperosmotic media (highly shrunken) was different from that due to mechanical stress (buckled or folded). Thus, the observed effect was attributable to the hyperosmotic conditions, and not to mechanical stress.

**DISCUSSION**

**Background to the Problem**

The literature on MB function in nonmammalian vertebrate erythrocytes has been a source of some confusion. The MB is generally understood to be of universal importance in erythrocyte morphogenesis (conversion of sphere to flattened ellipse; 2) but not in the continuing maintenance of cell shape.

The basic observations are as follows: (a) The normal developmental sequence for erythrocytes supports a morphogenetic role for MBs. Attainment of flattened, elliptical erythrocyte morphology is concomitant with MB biogenesis (1, 17, 24, 26). (b) In certain species (e.g., chicken, dogfish; 1, 4, 12), experimentally induced MB disassembly at 0°C does not affect the shape of mature erythrocytes. However, such treatment causes reversion to spherical morphology in immature cells (chicken; 1). (c) In some species, the number of MTs in the MB diminishes during erythrocyte maturation while typical erythrocyte morphology is retained (1, 26). Behnke (4) and Barrett and Dawson (1) thus concluded that the MB of at least some species is not responsible for erythrocyte shape maintenance. In the absence of direct evidence supporting a maintenance function, there is an implied possibility that MBs serve no continuing function in the mature erythrocytes of any species.

This possibility runs counter to the original impression of light microscopists who discovered the MB in stained cytolological preparations. Meves (20), in particular, noted that MBs were fibrous and flexible, and that their circumferential location in the plane of flattening was appropriate for a shape maintenance function. A maintenance role was also supported by Fawcett and Witebsky (15), who occasionally observed loops in stained MBs indicative of the required flexibility. In addition, MBs are present in the circulating erythrocytes of all nonmammalian vertebrates (including species with nonnucleated red cells; 11), for which Goniakowska-Witalinska and Witalinski (17) have demonstrated a general positive correlation between MB thickness (MT number in cross-section) and erythrocyte size suggestive of a mechanical role. Can the universal retention of such MBs simply be a matter of economy, with no continuing function?

**MB Function in Cell Shape Maintenance**

Much of the evidence cited either in favor of or against MB function in erythrocyte shape maintenance is indirect. Moreover, although the observed maintenance of erythrocyte shape after MB disassembly at 0°C appears to address the question directly, it is relevant only to the static conditions of microscopic examination. Erythrocytes are not designed for existence under such conditions, but are continually subjected to a variety of forces while moving in the bloodstream.

Our approach to studying MB function in mature erythrocytes thus differs from that of previous workers in two major respects: (a) we compared cells with and without MBs under identical conditions of temperature, and (b) we studied their
FIGURE 6  Effect of hyperosmotic stress on cells with and without MBs. 1 vol of cell suspension with or without MBs (colchicine) was mixed with 3 vol of 1.5 M sucrose, incubated for 5 min, then fixed by addition of glutaraldehyde to 1%. (a) Cells with MBs remain elliptical in contour. (b) Some of the cells without MBs shrivel (arrowheads). Phase-contrast microscopy. × 1,030.

TABLE III
Effect of Hyperosmotic conditions on cells with and without MBs

| Hyperosmotic solution | Experiment | Presence of MB | Shriveled cells |
|-----------------------|------------|----------------|-----------------|
| 1.5 M sucrose         | 1          | +              | 0.8             |
|                       |            |                | 19.9            |
| 1.5 M sucrose         | 2          | +              | 0.8             |
|                       |            |                | 12.0            |
| 1 M sucrose           | 3          | +              | 0.4             |
|                       |            |                | 11.8            |
| 1 M sucrose           | 4          | +              | 0               |
|                       |            |                | 6.1             |

Cell suspensions were mixed with sucrose solutions and incubated as described in Materials and Methods. Each percentage represents a minimum of 200 cells scored for shriveled versus smooth appearance.

responses to stress of the type which might be encountered in the circulation. The flow rate (~30 ml/min) used during cell fluxing was within physiological ranges (22), and the capillary tube diameter (400 μm, about the same as a “medium artery,” 25) was 20 times the longest cell dimension. However, we should point out that it was not our intention to mimic in vivo conditions, a near impossibility considering the lifetime of mechanical stress to which the cells are subjected in the animal.

In agreement with previous workers, we found that cells without MBs were usually normal in appearance. However, in the mechanical stress experiments, much greater percentages of cells without MBs were routinely found to be deformed. This was true regardless of the inhibitor used to produce cells lacking MBs at room temperature (colchicine, nocodazole), and regardless of the temperature at which such cells were produced (taxol) (Table I). Thus the observed differences were attributable to the MB, rather than specific inhibitor or temperature effects. Dogfish erythrocytes, like erythrocytes in general, are highly deformable. As observed in flowing Ringer’s under the coverslip, the cells undergo extensive shape changes when squeezed between other cells, but quickly recover normal shape. Therefore, we assume that many of the cells initially responded by deformation, with the MB bringing about the rapid reversion to normal shape in most of these cells. Such a function is readily extrapolated to the in vivo situation, in which there is mechanical interaction with other cells and blood vessel walls. However, the MB could also act to inhibit initial deformation or prevent structural damage. The evidence presented here for MB function in morphological stability opens these questions for further study.

The continuing MB function indicated by our data is a different kind of “maintenance” from that addressed by previous workers (1, 4). It is maintenance of the mature erythrocyte shape against deformation by external forces, as opposed to maintenance of the differentiated state. This distinction was alluded to by Behnke in his review (5). Once it is made it becomes clear that some of the confusion concerning MB function derives from the fact that different investigators have used the term “maintenance” to describe different cellular properties.

Our results are in agreement with previous observations on maintenance of the differentiated state in the absence of MBs. Although we do not know what structural components are responsible for this, the following observations may be of value. Ghosts prepared by osmotic lysis of cells without MBs remain generally elliptical and smooth in contour (unpublished observation). However, Triton X-100 extraction of erythrocytes without MBs produces a collapsed cytoskeleton (12). Therefore, membrane components extracted by Triton X-100, such as phospholipids or proteins analogous to human erythrocyte band 3 (8), may play a role. Such proteins appear to be extracted from the dogfish erythrocyte ghost by Triton X-100 (3). It is also possible that there is a morphological contribution by other cytoskeletal elements such as intermediate filaments (18) which may be labile under our Triton X-100 extraction conditions.

Mechanism of MB Function

By what mechanism might the MB restore normal shape after deformation and/or inhibit initial deformation? We approached this question in the current work by making use of pointed dogfish erythrocytes that appear when cell suspensi-
sions are incubated at room temperature for several hours, as reported earlier (12). In lysed samples of these suspensions, pointed cytoskeletons containing pointed MBs are observed. The perfusion experiments presented here demonstrate that the MBs have direct morphological correspondence to cells with respect to point location and number. The ability to generate cells with and without MBs allowed us to test whether the MB was the effector of point formation. The answer is clear: as shown in Table II, significant numbers of pointed cells were obtained only when MBs were present. As in the case of the mechanical stress experiments, the result was independent of the particular inhibitor used to generate cells without MBs.

Although we do not yet understand the mechanism by which the anomalous MBs are generated, it is apparent that the resulting point on the MB causes the cell to become pointed. We can interpret this effect based upon our current model for the dogfish erythrocyte cytoskeleton (Fig. 7), in which the primary elements are the MB and the cell surface-associated cytoskeleton (SAC). Since the MB abuts the SAC, it must be able to deform the SAC (and the entire membrane) from within; conversely, the SAC must be able to adopt to the change in MB shape.

In the normal flattened, elliptical erythrocyte, SAC shape coincides with that of the continuous elliptical MB. If the cell (and therefore SAC) were temporarily deformed by an external force, what properties of the MB would account for rapid restoration of normal shape? Generation of the points, which form at sites of MB breakage, offers a clue: once broken, the curved MT bundles tend to linearize (Fig. 4). Isolated MBs behave similarly: when transsected, they quickly open outward into a much more linear configuration (6, 9). When incubated with protease, straight, tangential fibers (MT bundles) strip away from the MB surface. All of these observations are most simply explained by release of internal strain (9). Treatment of cytoskeletons with various agents (6, 9–11) causes MBs to circularize, indicating equalization of strain distribution. Spontaneous redistribution of strain would provide the means by which MBs quickly revert to equilibrium shape within mechanically perturbed cells in vivo. We believe that this is the mechanism whereby the MB acts as a flexible frame within the SAC.

The hyperosmotic stress experiments presented here provide further in vivo evidence that the MB acts as a frame for the SAC and therefore for the cell. Many erythrocytes lacking MBs shrivel when exposed to hyperosmotic solutions, whereas those with MBs retain a relatively smooth, elliptical contour. The MB prevents the SAC from buckling and collapsing inward as the cells lose volume.

The MB function proposed, involving strain in a MT bundle, would likely be independent of the mechanism of MB assembly and of MT polarity. Thus it could apply to the MBs of diverse species and cell types, including those of invertebrate erythrocytes containing multiple microtubules organized by centrioles (21), and those of vertebrate erythrocytes and thrombocytes in which MB-associated centrioles are not apparent (such as the dogfish; Fig. 4). It would be compatible with the proposal that some MBs in erythrocytes of an individual animal may contain MTs of only one polarity and others of both polarities (14). We believe that our general
model of a strained MB frame within a SAC under tension (Fig. 7) applies to all cells in which MBs occur, with variations in the specific properties of the SAC and membranes of different cell types.

We wish to thank Dr. M. Suffness of the Natural Products Branch, Division of Cancer Treatment, National Cancer Institutes for providing taxol, and Mr. Doug Dressel of the Marine Biological Laboratory for excellent technical assistance in obtaining the scanning electron micrographs. We are also indebted to Dr. Sidney K. Pierce, University of Maryland and the Marine Biological Laboratory, for advice on the osmotic experiments and for the osmolality measurements. This work was supported by City University of New York PSC-BHE grants, Nos. 6-62156 and 6-63177, and National Science Foundation grant No. PCM-8107195.

Received for publication 11, November 1983, and in revised form 21, February 1984

REFERENCES

1. Barrett, L. A., and R. P. Dawson. 1974. Avian erythrocyte development: microtubules and the formation of disc shape. Dev. Biol. 36:72-81.
2. Barrett, L. A., and S. L. Schenck. 1972. The development of avian red cell shape. J. Exp. Zool. 182:1-14.
3. Barrett, D., R. Carlson, G. Scheitl, and W. D. Cohen. 1982. The cytoskeletal system of nucleated erythrocytes. II. Presence of a high molecular weight calmodulin-binding protein. J. Cell Biol. 95:278-284.
4. Behnke, O. 1970. Microtubules in disk-shaped blood cells. Int. Rev. Exp. Pathol. 9:1-92.
5. Bertolini, B., and G. Monaco. 1976. The microtubule marginal bundle of the new erythrocyte. Observations on the isolated band. J. Ultrastruct. Res. 54:67-67.
6. Cavanaugh, G. M., editor. 1975. Formulae and Methods VI of the Marine Biological Laboratory, Woods Hole, Mass. 70.