Shape-changing agents produce abnormally large platelets in a hereditary "giant platelets syndrome (MPS)"

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Geometries of platelets in citrated PRP obtained from normal donors (17) and donors (5) with a hereditary dominant giant platelet syndrome, herein referred to as "Montreal platelet syndrome" (MPS), are compared. The measured geometric axial ratio (r_p = thickness/diameter) is used to classify platelet morphologies into three groups: discocytes ($r_p < 0.5$), disco-echinocytes ($r_p = 0.5$ to 0.9), sphero-echinocytes ($r_p > 0.9$). MPS discocytes are normal sized; however, MPS sphero-echinocytes and disco-echinocytes have mean volumes approximately two times larger than normal. It is demonstrated that these larger-than-normal sized MPS platelets can be produced directly from MPS discocytes by treatment with agents known to induce platelet shape change (adenosine diphosphate, thrombin, and incubation at 4° C). Treatment of platelets obtained from normal donors which have been resuspended in MPS PPP with ADP or incubation at 4° C causes the formation of normal-sized disco-echinocytes and sphero-echinocytes. The diameters of MPS disco-echinocytes are identical to the diameters of MPS platelets observed on peripheral blood smear, whereas those of MPS sphero-echinocytes are ~20% lower. It is suggested that the appearance of abnormally large platelets in MPS is related to a defect in the mechanism which regulates platelet size and shape during shape change. (J LAB CLIN MED 93:154, 1979.)

Abbreviations: Montreal platelet syndrome (MPS), Bernard-Soulier syndrome (BSS), platelet-rich plasma (PRP), platelet-poor plasma (PPP)

Circulating unactivated platelets for two hereditary "giant" platelet syndromes, BSS and a poorly characterized hereditary giant platelet syndrome (HGPS; hereafter referred to as Montreal platelet syndrome (MPS)), are normal-sized even though on peripheral blood smear the platelets are 1.6 to 1.7 times larger than normal.[1] On the basis of this observation it has been suggested that the giant size of these platelets arises during the preparation of the blood smear.[1] Here we show that in MPS, abnormally large platelets can be produced directly by treatment with stimuli known to produce shape change in normal platelets. The appearance of abnormally large platelets in MPS as a result of an abnormal platelet shape change is to be distinguished from other giant platelet syndromes where it appears that giant-sized unactivated platelets may indeed be present, as for example in May-Hegglin's disease.[2]

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Table I. Mean diameters ($\bar{d}$), thickness ($\bar{t}$), axial ratio ($\bar{r}_p$), and volumes ($\bar{V}_D$) for discocytes from MPS and normal donors

Donor	$\bar{d}$ (μm)	$\bar{t}$ (μm)	$\bar{r}$	$\bar{V}_D$ (μm^3)
Normal	3.1 ± 0.3*	1.0 ± 0.2*	0.3 ± 0.1*	5.5 (4.0-7.6)†
Unaffected MPS:				
F. T.	3.1	0.9	0.3	4.7
M. B.	2.9	1.1	0.4	5.0
Affected MPS:				
M. T. (1)‡	2.7	1.0	0.4	4.3
I. T. (3)	3.0	1.2	0.4	6.0
L. T. (3)	2.7	1.0	0.4	4.2
P. T. (2)	2.7	1.1	0.4	4.5
I. B. (1)	2.9	1.3	0.4	6.0

*Values are reported as mean ± 2 S.D. units with data obtained from 17 different normal donors.
†Numbers in parentheses give lowest and highest value measured.
‡Numbers in parentheses give the numbers of times the patient's discocytes were examined. More than a total of 90 discocytes were examined in each case with the exception of M. T. (21) and I. T. (23). Variation in $\bar{d}$ and $\bar{r}_p$ was less than 4% and for $\bar{V}_D$ and $\bar{t}$ less than 10%.

Methods and materials

Case histories.* Normal donors were chosen from healthy men and women between the ages of 20 and 35 years. Donors with the "giant" platelet syndrome were taken from a family whose case was first described by Lacombe and d'Angelo.[3] This syndrome is characterized by dominant autosomal inheritance, the appearance of giant platelets on peripheral blood smears, a prolonged bleeding time, a greatly reduced platelet count (<10,000 to 150,000 μl^{-1}), spontaneous platelet aggregation and normal clot retraction, and thromboplastin formation. Ristocetin-induced aggregation is normal for MPS, but thrombin-induced aggregation is low to absent (Milton, Tang, and Frojmovic, submitted). We have designated this syndrome as MPS to distinguish it from other hereditary dominant thrombocytopenic macrothrombopathias. The present study examines platelet morphologies for members of three generations of a family affected with MPS: an affected mother (M. T., 65 years old), her unaffected husband (F. T., 65), her two affected daughters (I. T., 34; L. T., 37), her affected son (P. T., 29), her affected grand-daughter (I. B., 13; daughter of L. T.), and her unaffected grandson (M. B., 12; son of L. T.). The clinical descriptions of M. T., I. T., L. T., and P. T. have been given previously.[3]

Preparation of PRP and activated platelets. Blood was drawn by venipuncture into 3.8% citrate (1 vol to 9 vol blood), and PRP was prepared as described previously.[1, 4] For discocyte and sphero-echinocyte size measurements, PRP was fixed with 4 vol of 1.3% glutaraldehyde in Tyrode's buffer, pH 7.4, at 37° C.[1, 4] Shape-changed platelets were produced either by treating PRP with 10 μM ADP (Sigma Chemical Co., St. Louis, Mo.)[5] or 0.2 U/ml human thrombin (specific activity: 4000 to 5000 NIH U/mg; Sigma) or by exposing PRP to 4° C.[6] Samples were removed and fixed with glutaraldehyde 30 sec after the addition of ADP or thrombin and after 30 min incubation at 4° C.

Preparation of PPP and platelet resuspension. PPP was prepared from citrated PRP by centrifuging the latter at 1900 × g, 37° C, for 30 min. PPP was allowed to incubate at 37° C for an additional 30 min prior to use, to remove residual ADP. Since platelets pelleted from citrated PRP could not be resuspended, the following procedure was adopted because it did not alter the fraction of discocytes or the geometry of activated and unactivated platelets: 1 vol of blood was collected into 6 vol of ACD (acid citrate dextrose: 85 mmol trisodium citrate, 72 mmol citric acid, 111 mmol dextrose) and allowed to stand at 37° C for 30 min. ACD-PRP was prepared by centrifuging at 150 × g, 37° C, for 15 min and a platelet pellet from ACD-PRP by centrifuging at 1900 × g, 37° C, for 30 min. Platelet pellets prepared from ACD-PRP were easily resuspended into citrated PPP, pH 7.4.

Classification of platelet morphologies. Freely rotating platelets as viewed under phase-contrast microscopy (Zeiss universal microscope, magnification 800×; Carl Zeiss, Inc., New York, N. Y.) were classified into one of three groups, using the terminology introduced by Bessis.[7] Unacti-

*These experiments were performed according to the principles of the Declaration of Helsinki, and informed consent was obtained.

Table II. Comparison of platelets in normal and MPS PRP

Donor	% of each platelet type			$\bar{V}_D$ (μm^3)	$\bar{V}_{DE}$ (μm^3)	$\bar{V}_{SE}$ (μm^3)	$\bar{V}_T$* (μm^3)
	Discocytes	Disco-echinocytes	Sphero-echinocytes				
Normal (4)†	75-95	5-25	<2	5.3 (4.5-6.1)‡	6.5 (5.5-7.0)	3.9 (3.1-4.7)	5.0-6.0
MPS (3)	20-85	15-60	1-20	4.9 (4.2-6.0)	10.2 (8.5-11.1)	7.8 (6.6-9.8)	5.0-10.0

*$\bar{V}_T$ = platelet volume.
†Normal donors were randomly selected from those whose $\bar{V}_D$ fell between 4.0 and 6.0 μm^3.
‡Numbers in parentheses give lowest and highest value measured. The ranges for % platelet type, $\bar{V}_{DE}$, and $\bar{V}_T$ arise because of intra- and inter-donor variations; for the others the ranges are essentially due to inter-donor variations.

vated, disc-shaped platelets (discocytes) were characterized as oblate ellipsoids with a diameter (d), thickness (t) and calculated axial ratio (r_p = t/d). Disco-echinocytes were identified as discocytes which possessed pseudopods and/or as swollen discocytes with r_p = 0.5 to 0.9 (less than 0.5% of discocytes have $r_p \geq 0.5$ in a normal, unactivated platelet population). Spherically-shaped platelets ($r_p \geq 0.9$), typically possessing many pseudopodia, were classified as sphero-echinocytes. Less than 1% of the platelets in normal and MPS PRP could not be placed into one of these three groups. The term "echinocyte" is used to describe both disco-echinocytes and sphero-echinocytes.

Measurement of geometric parameters. Freely rotating platelets were filmed under phase-contrast microscopy (magnification 512×) with a 16 mm Beaulieu movie camera utilizing a fine-grain film, and the film was projected onto a screen for analysis (over-all magnification 8000×). Film analysis and criteria for selecting an edge-on orientation and measuring t and d are the same as previously reported.[4] Except where noted, at least 80 platelets were measured for the determination of mean geometric parameters. Geometric parameters of activated discocytes were measured as for unactivated discocytes. The main body of the sphero-echinocytes, i.e., excluding pseudopodia, was approximated as a sphere, with the diameter chosen as for the discocyte (mid-point between primary circle and outside of first diffraction ring). Measurement of diameters of commercial latex particles indicated that diameters measured in this way agree to within 5%.[1] Discocyte (V_D) and disco-echinocyte (V_{DE}) volumes were calculated as $V = \frac{\pi}{6} d^2 t$; that of sphero-echinocyte (V_{SE}) was calculated by the same formula with t = d.

Mean volume of platelets in PRP (V_T) was calculated from the relation

$$\bar{V}_T = f_D\bar{V}_D + f_{DE}\bar{V}_{DE} + f_{SE}\bar{V}_{SE}$$

where $\bar{V}_D$, $\bar{V}_{DE}$, $\bar{V}_{SE}$ are, respectively, the mean volume of discocytes, disco-echinocytes, and sphero-echinocytes and f_D, f_{DE}, f_{SE} are the fractions of each cell type present ($f_D + f_{DE} + f_{SE} \approx 1.0$).

Results

MPS discocytes. Preliminary size measurements of discocytes for donor I. T. have been discussed previously.[1] Table I compares discocyte sizes for all affected and unaffected members of the MPS family with those obtained from normal donors. There was a tendency for MPS discocytes to have smaller volumes than normal and to be slightly more swollen than normal; that is, $\bar{d}$ was smaller and $\bar{t}$ was larger than normal. In order to remove the possibility that the geometries of platelets on shape change may be a function of discocyte size, geometries of MPS shape-changed platelets were compared only to those of normal donors randomly selected from persons who had similar discocyte mean volume.

MPS PRP prior to shape change. Table II compares platelet populations from normal and MPS PRP. MPS PRP typically had a greater proportion of nondiscoid shaped platelets, i.e., disco-echinocytes and sphero-echinocytes. The volumes of the nondiscoid MPS platelets were from 1.7 to 2.3 times larger than would be anticipated for a normal donor with similar-sized discocytes. This observation suggests that it is the activated

Table III. Comparison of geometries of normal and MPS sphero-echinocytes prepared by cold. thrombin, and ADP*

Treatment	$\bar{d}$ (μm)	$\overline{V}_{SE}$ (μm^3)	$\overline{V}_{SE}/\overline{V}_D$
ADP:			
Normal (5)†	1.9 (1.8-2.0)‡	3.8 (3.1-4.6)	0.7 (0.6-1.0)
M. T.§	1.8	3.1	0.6
MPS (5)	2.4 (2.2-2.5)	8.2 (6.6-10.0)	1.7 (1.3-2.3)
Cold:			
Normal (3)†	1.9 (1.8-2.0)	3.8 (3.5-4.1)	0.7 (0.6-0.8)
MPS (3)	2.3 (2.2-2.4)	7.2 (6.2-8.6)	1.5 (1.4-1.6)
Thrombin:			
Normal (1)†	2.0	4.4	0.7
MPS (3)	2.3 (2.2-2.6)	7.4 (6.2-9.6)	1.5 (1.4-1.6)

*In all cases reported here the number of sphero-echinocytes prior to treatment was less than 15%of those present after treatment.
†Normal donors were randomly selected from those whose $\overline{V}_D$ fell between 4.0 and 6.0 μm^3.
‡Numbers in parentheses give lowest and highest value measured. The ranges represent essentially variations in observations made in a number of different donors in each population.
§Normal MPS family member.

platelets (i.e., echinocytes) which are giant-sized. We next demonstrated that these larger-than-normal sized platelets did not represent a distinct subpopulation of platelets but probably have arisen via shape change from the normal-sized discocytes.

MPS sphero-echinocytes. Table III compares sphero-echinocytes obtained from normal and MPS donors. MPS sphero-echinocytes were 1.8 to 2.2 times larger than normal. Although in normal platelet shape change, sphero-echinocyte formation typically occurred with a slight decrease in mean volume compared to discocytes ($\overline{V}_{SE}/\overline{V}_D < 1$), MPS sphero-echinocytes had mean volumes larger than MPS discocytes ($\overline{V}_{SE}/\overline{V}_D > 1$).

Fig. 1 compares the size distribution of MPS sphero-echinocytes before and after treatment of PRP with ADP. A Student's t test was performed and demonstrated that these two distributions are not significantly different (probability is $0.9 > p > 0.5$). Therefore it appears that the larger-than-normal MPS sphero-echinocytes present in MPS-PRP have arisen via abnormal shape change of normal-sized discocytes.

MPS disco-echinocytes. A preliminary kinetic analysis of the changes in normal platelet morphologies following induction of platelet shape change suggested that disco-echinocytes represent intermediate forms between the unactivated discocyte and the fully shape-changed sphero-echinocyte (Milton, Glushak, and Frojmovic, unpublished data).

Fig. 2 shows the changes in MPS platelet morphologies as a function of time following the addition of 10 μm ADP. The time-dependent trends in f_D, f_{DE}, f_{SE} are consistent with the model[8]:

$$\text{Discocyte} \longrightarrow \text{disco-echinocyte} \longrightarrow \text{sphero-echinocyte}$$

Therefore we have demonstrated that platelets in MPS PRP are either normal-sized discocytes or larger-than-normal sized platelets which probably have arisen via shape change from these discocytes.

Location of MPS shape-change defect. Platelets obtained from a normal donor were resuspended in normal and MPS PPP. The results in Table IV show that under these conditions, ADP and cold caused the formation of normal-sized echinocytes. Therefore the MPS shape-change defect resides at the level of the platelet.

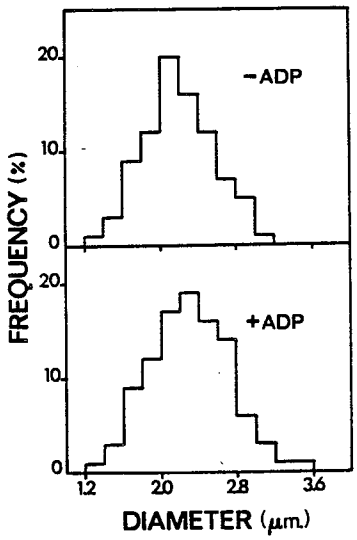

Fig. 1. Histogram of diameter for sphero-echinocytes in MPS PRP and in MPS PRP 30 sec after addition of ADP (final concentration ~10 μM). MPS donor was P. T. Prior to addition of ADP there were 2% sphero-echinocytes in the PRP, and after the addition of ADP 35%. Similar trends were observed for the other MPS donors.

Table IV. Geometries of echinocytes produced from normal platelets suspended in normal and MPS PPP by ADP and cold

Agent	*Suspending medium*	$\bar{d}$ (μm)	$\overline{V}_{DE}$ (μm³)	$\overline{V}_{SE}$ (μm³)
ADP	Normal PPP	1.8	4.8	3.1
	MPS PPP	1.7	4.6	2.9
Cold	Normal PPP	1.8	4.1	3.5
	MPS PPP	1.8	4.2	3.3

Discussion

We have demonstrated that for a particular hereditary giant platelet syndrome (MPS) in which the discocytes are normal-sized, larger-than-normal sized platelets can be produced with shape-changing agents. The fact that both physical and biochemical stimuli produce large platelets in MPS suggests that the MPS platelet defect must be at the level of one of the fundamental mechanisms which control platelet shape change.

Morgenstern and co-workers[9, 10] have suggested that during normal platelet shape change, a portion of the invaginated plasma membrane evaginates. MPS platelets possess a normal amount of invaginated plasma membrane.[11] This observation indicates that the large size of MPS shape-changed platelets may reflect a subtle flaw in the way in which plasma membrane is reorganized during platelet shape change.

The data in Table II show that platelet mean volume, $\overline{V}_T$, for MPS PRP varies from 1 to 2 times normal. In particular, $\overline{V}_T$ becomes large as f_D decreases, since it is the nondiscoid platelets which are larger than normal-sized. Although we have shown that large-sized shape-changed platelets are derived from normal-sized discocytes in MPS, the reasons why untreated MPS PRP can contain a relatively high proportion of nondiscoid shaped platelets are not clear. Addition of MPS PPP to normal PRP or resuspending normal

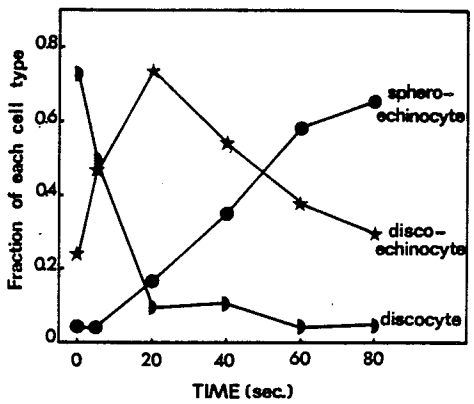

Fig. 2. Platelet shapes as a function of time following the addition of ADP (final concentration ~10 μM) to MPS PRP. At the indicated times, 0.05 ml was withdrawn and fixed with glutaraldehyde, and platelet shapes were classified by examination under phase-contrast microscopy. In each case a total of 400 platelets was counted. MPS donor was I. T. Identical trends were obtained with P. T.

Table V. Comparison of diameters of normal and MPS discocytes, disco-echinocytes, and sphero-echinocytes in PRP and platelets on blood smear

	Mean diameter				
Donor	*Blood smear*	*Discocyte*	*Disco-echinocyte**	*Sphero-echinocyte*	*Spherocyte*
Normal (3)	1.8	3.0	2.6-2.4	1.8	3.2
MPS:					
I. T.	3.2	3.0	3.2-2.8	2.5	3.2
P. T.	3.0	2.6	3.0-2.6	2.3	3.1

*The value on the left is the diameter measured in untreated PRP and that on the right is the one measured in cold- and ADP-treated PRP. For disco-echinocytes at least 40 platelets were measured.

platelets in MPS PPP does not alter f_D. Thus MPS plasma does not appear to contain a factor which induces platelet shape change. It is possible that MPS platelets are more sensitive to surface activation in circulation. Whatever the reason, the tendency of MPS PRP to have a high proportion of nondiscoid platelets may be related to the nature of the MPS shape-change defect.

A few investigators have used Coulter Counter measurements to compare $\overline{V}_T$ for platelets from normal and giant platelet syndrome donors. Mean platelet volumes were 1.8 to 1.9 times larger for donors with a hereditary thrombocytopenia with normal platelet life-span[12–13] and 2.3 times larger than normal for a donor with a hereditary thrombocytopenia with associated deafness.[14] These volumes compare well with those observed for MPS donors when shape change has occurred, i.e., when f_D is small. However, it must be emphasized that the similarity of these independent observations may be fortuitous, since the morphology of the platelets studied by the Coulter Counter was not reported. Mean volumes of MPS platelets are abnormally large only after shape change has occurred. It is quite possible that the diluting solutions used in Coulter Counter measurements induces platelet shape change in giant platelet syndromes. Indeed, we have observed that for some of the MPS donors, f_D for MPS PRP can be reduced by simply diluting it with isotonic Tyrode's solution (f_D for normal PRP was not altered by this

procedure). It is also possible that the platelet mean volumes observed in other giant platelet syndromes indicate the presence of abnormally large discocytes. These observations serve to emphasize the need for careful morphological microscopic analysis in working with platelets from giant platelet syndrome donors.

We have suggested that the giant size of MPS platelets on blood smear arises during the preparation of the blood smear.[1] Table V compares diameters of normal and MPS platelets on blood smear with the diameters of discocytes, disco-echinocytes, and sphero-echinocytes. For normal platelets it is clear that shape change must have occurred during preparation of the blood smear. Although the diameters of MPS sphero-echinocytes are ~20% to 30% smaller than those observed on blood smear, the diameters of MPS disco-echinocytes in untreated PRP are essentially identical to those observed on blood smear, with only a small decrease observed in fully activated PRP. Thus the giant size of MPS platelets on blood smear is consistent with the sizes we have reported here for MPS platelets which have undergone partial shape change to form disco-echinocytes. However, it is possible that MPS platelets on blood smear have morphologies distinct from those encountered in shape change. In the accompanying paper,[11] we show that by an appropriate choice of hypotonic media, platelets can be transformed into large, smooth spherical forms (spherocytes) and that spherocytes represent the largest size that platelets can attain without lysis. As can be seen from Table V, MPS spherocyte diameters also agree well with the diameters observed on blood smear. Lastly, our observations do not completely exclude the possibility that the diameter of platelets observed on blood smear depends, in part, on characteristics of the cell other than the cell volume. For example, characteristics of the cell membrane or other properties of the cell may influence the degree to which the cell spreads when it is in contact with glass.

Platelet shape change is generally regarded as the initial event for the participation of platelets in hemostasis and thrombosis.[15, 16] The fact that this process is impaired in MPS suggests that there may be associated functional defects. Indeed we have found that ADP-and thrombin-induced aggregation of MPS platelets is abnormal (Milton, Tang, and Frojmovic, submitted). Our studies with MPS suggest the possibility that the appearance of giant platelets on blood smear points to a platelet shape-change defect, and this may be useful for interpreting the etiology of and functional defects in giant platelet syndromes.

It is hoped that other investigators working on different giant platelet syndromes will determine whether or not there is an associated morphologic shape-change defect.

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REFERENCES

1. Frojmovic MM, Milton JG, Caen JP, et al: Platelets from "giant platelet syndrome (BSS)" are discocytes and normal sized. J LAB CLIN MED **91:**109, 1978.
2. Lechner K, Breddin K, Moser K, et al: May-Hegglinsche Anomalie. Acta Haematol **42:**303, 1969.
3. Lacombe M and d'Angelo G: Etudes sur une thrombopathie familiale. Nouv Rev Fr Hematol **3:**611, 1963.
4. Frojmovic MM and Panjwani R: Geometry of normal mammalian platelets by quantitative microscopic studies. Biophys J **16:**1071, 1976.
5. Born GVR: Observations on the change in shape of blood platelets brought about by adenosine diphosphate. J Physiol **209:**487, 1970.
6. White JG and Krivit W: An ultrastructural basis for the shape changes induced in platelets by chilling. Blood **30:**625, 1967.

7. Bessis M: Red cell shapes: an illustrated classification and its rationale. *In:* Red Cell Shape, Bessis M, Weed RI, and LeBlond PF, editors. New York, 1973, Springer-Verlag.
8. Frost AA and Pearson RG: Kinetics and Mechanism. New York, 1961, John Wiley & Sons, Inc., pp. 160-169.
9. Morgenstern E and Kho A: Morphometrische Untersuchungen an Blutplattchen: Veranderungen der Plattchenstruktur bei Pseudopodien-Bildung und Aggregation. Cytobiologie **15:**233, 1977.
10. Morgenstern E, Walter E, and Webber E: Platelet membranes during various functional states. *In:* Erythrocytes, Thrombocytes, Leukocytes, Gerlach E, Moses K, Deutsch E, and Wilmans W, editors. Stuttgart, 1973, Georg Thieme Publishers, pp. 240-243.
11. Milton JG and Frojmovic MM: Invaginated plasma membrane of human platelets: evagination and measurement in normal and "giant" platelets. J LAB CLIN MED **93:**162, 1979.
12. Murphy S, Oski FA, Maiman JL, et al: Platelet size and kinetics in hereditary and acquired thrombocytopenia. N Engl J Med **286:**499, 1972.
13. Murphy S: Intrinsic platelet defects in hereditary thrombocytopenia. Ann NY Acad Sci **201:**421, 1972.
14. Eckstein JD, Filip DJ, and Watts JG: Hereditary thrombocytopenia, deafness and renal disease. Ann Intern Med **82:**639, 1975.
15. White JG: Shape change. Thromb Diathes Haemorrh Suppl **60:**159, 1974.
16. Ehrman M, Toth E, and Frojmovic M: A platelet procoagulant activity associated with platelet shape change. J LAB CLIN MED **92:**393, 1978.