

## Platelet Function in a Patient with Thrombasthenia

By MARJORIE B. ZUCKER, JAMES H. PERT AND MARGARET W. HILGARTNER

**A** PLASMA CLOTTING FACTOR is deficient in most of the "experiments of nature" that have helped to further our understanding of the mechanism of blood coagulation and hemostasis, but in thrombasthenia the defect lies in the platelets which are unable to promote clot retraction. Thrombasthenic patients usually have a mild to moderately severe hemorrhagic diathesis, with a prolonged bleeding time. This rare congenital disorder, also known as Glanzmann's or Glanzmann-Naegeli's disease, is distinguished from thrombocytopathy in which the platelets' faulty thromboplastic function is the major defect while clot retraction is normal. Other aspects of the differential diagnosis of platelet defects have been reviewed elsewhere.<sup>1-3</sup> We are presenting here observations made over the past five years on a patient with thrombasthenia. Some of these studies have been mentioned briefly in other publications.<sup>4-7</sup>

### CASE HISTORY

The patient (New York Hospital—No. 793146), now 10 years old, developed a huge local ecchymosis following circumcision soon after birth. He tended to form hematomas following trauma and often had scattered petechiae over the body. Epistaxis necessitated hospitalization and transfusions at the age of six, but has not been a problem recently. The patient has a cardiac murmur presumably due to mild aortic stenosis. Electrocardiograms suggested incomplete right bundle branch block, and phonocardiograms revealed an early systolic murmur and split second sound similar to that heard with aortic stenosis. Heart size was normal. The only clinical symptoms related to the patient's hemorrhagic tendency, which has somewhat limited his physical activity. The parents and three siblings had shown no tendency to excessive bleeding.

### METHODS

Platelet-rich plasma (PRP) was prepared by slow centrifugation of blood collected in one-ninth volume 0.11 M sodium citrate or 0.027 M ethylenediamine tetraacetate (EDTA); one-seventh volume acid-citrate-dextrose (ACD) formula A; or one-fiftieth volume heparin (10 mg./ml.). References to specific methods are given in Table 1. Concentrations of drugs, etc.,

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*From the American National Red Cross Research Laboratories; Department of Pathology, New York University School of Medicine; Department of Medicine, George Washington University School of Medicine; and Department of Pediatrics, Cornell University Medical College.*

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MARJORIE B. ZUCKER, PH.D.: Senior Research Associate, American National Red Cross Research Laboratories, Eastern Division, and Associate Professor of Pathology, New York University School of Medicine, New York, N. Y. JAMES H. PERT, M.D.: Research Director, American National Red Cross Blood Program, and Assistant Clinical Professor of Medicine, George Washington University School of Medicine, Washington, D. C. MARGARET W. HILGARTNER, M.D.: Instructor in Pediatrics, Cornell University Medical College, and Attending Pediatrician, Department of Pediatrics, New York Hospital, New York, N. Y.

represent final values. Phospholipase C was obtained from Worthington Biochemical Corp., and Polybrene from Abbott Laboratories.

### *Platelet Suspensions*

Platelets from EDTA anticoagulated blood were washed once in 1 per cent ammonium oxalate solution and twice in isotonic saline. Suspensions containing about 400,000 platelets/mm.<sup>3</sup> were used for Fi-Tests and immunofluorescent staining; suspensions containing over  $20 \times 10^6$  platelets/mm.<sup>3</sup> were frozen and thawed 3 to 5 times and centrifuged at 10,000 g for 10 minutes to prepare the supernatant used for immunoelectrophoresis and additional Fi-Tests.

Fi-Test reagent (latex beads coated with antiserum to human fibrinogen) and latex beads coated with serum from nonimmunized rabbits, for use as a control, were kindly provided by Dr. Kingdon Lou of the Hyland Laboratories, Los Angeles, California. Samples to be tested for fibrinogen were serially diluted with isotonic saline containing 0.02 M imidazole buffer, pH 7.2. Antiserum to human fibrinogen, used for immunoelectrophoresis, gave a single line against human plasma or purified fibrinogen and none against human serum. A similar antiserum tagged with fluorescein was used for immunofluorescent staining of platelet fibrinogen. Specificity for fibrinogen was shown by absorption of the conjugated antiserum with a preparation of highly purified human fibrinogen (provided by Dr. Alan J. Johnson). Drops of washed platelet suspensions were air-dried on glass slides. The slides were fixed for 10 minutes in acetone, washed for 5 minutes in 0.15 M saline containing 0.02 M phosphate buffer at pH 7.0, and covered with fluorescent antiserum for 30 minutes in a moist chamber at room temperature, followed by two 5-minute washes in buffered saline. The slides were examined with a Zeiss microscope equipped with an Osram HBO 200 watt lamp and a dark-field immersion condenser. We are very grateful to Dr. Pierre Vassalli of the Department of Pathology, New York University Medical Center, for the immunofluorescence studies, and to Dr. Gerard Sokal of the University of Louvain, Belgium, for carrying out a preliminary study.

### RESULTS

The patient's platelet count was 200,000/mm.<sup>3</sup> in 1958; since 1961, counts as low as 100,000/mm.<sup>3</sup> have been noted. Studies by four competent blood coagulation specialists demonstrated normal values for clotting time, prothrombin time, plasma fibrinogen concentration, and thromboplastin generation test (TGT), using the patient's plasma (Hicks-Pitney)<sup>8</sup> or plasma and serum (Biggs and Douglas).<sup>8</sup> Abnormal findings were bleeding time over 15 minutes, positive tourniquet test, no clot retraction in whole blood at 1 hour and only slight retraction after 3 hours at 37 C., and prothrombin consumption between 40 and 70 per cent (normal over 80 per cent). Washed platelets gave abnormal results in the TGT in one of four tests.

Retraction of clots produced by adding thrombin to the patient's citrated PRP at 37 C. was only 1+ at 2 hours, compared with 4+ for normal PRP in 20 minutes. Addition of magnesium chloride (final concentration 0.01 M), but not calcium chloride, just prior to thrombin had no noticeable effect at 20 minutes, but retraction was 2+ at 1 hour and 3+ at 2 hours. Further results are presented in Table 1.

Immunoelectrophoresis with fibrinogen antiserum revealed a single precipitin line with solutions containing more than 0.5  $\mu$ g. fibrinogen/mm.<sup>3</sup>, and with the supernatant from frozen and thawed suspensions containing 11.1 or  $22.2 \times 10^6$  normal platelets/mm.<sup>3</sup>, but none for platelet concentrations of 2.8 or  $5.5 \times$

Table 1.—Comparison of Normal and Thrombasthenic Platelets

|  | Patient<br>N. Y. H. =<br>793146 | Normal<br>Values             | Method<br>Reference |
|--|---------------------------------|------------------------------|---------------------|
| <i>Adenosine Triphosphate in Platelets</i><br>( $\mu\text{M.}$ per $10^9$ platelets)                               |                                 |                              | 9, 10               |
| EDTA PRP unincubated   | 75.5                            | $44.4 \pm \text{S.D. } 11.4$ |                     |
| EDTA PRP + $\text{MgCl}_2$ , 30 min. 37 C.   | 78.4                            | 37                           |                     |
| EDTA PRP + $\text{MgCl}_2$ + thrombin, 30 min.<br>37 C.  | 48.6                            | About 18                     |                     |
| <i>Glyceraldehyde-3-Phosphate-Dehydrogenase</i><br>(Bucher units per $10^8$ platelets)                             |                                 |                              | 5                   |
| Washed platelets   | 3.0                             | 2.2, 3.3                     |                     |
| <i>Platelet Adhesiveness to Connective Tissue</i><br>(C. T.)   |                                 |                              | 6                   |
| Citr. PRP applied to mesenteric fibers   | ++++                            | ++++                         | Observation         |
| <i>Platelet Adhesiveness to Glass</i>  |                                 |                              |                     |
| Citr. PRP applied to glass slides  | 0                               | ++++                         |                     |
| <i>Platelet Adhesiveness Test</i>  |                                 |                              | 11                  |
| Citr. blood passed through glass bead<br>column  | 10%°                            | over 35%                     |                     |
| <i>Platelet Aggregation</i>  |                                 |                              |                     |
| Native PRP (just before clotting)  | 0                               | ++++                         | 12                  |
| Heparinized PRP + ADP ( $1 \mu\text{M./L.}$ )  | 0                               | ++++                         | 6                   |
| Heparinized PRP + ADP + $\text{MgCl}_2$<br>8–12 mM./L.   | 0                               | ++++                         | 6                   |
| Heparinized PRP + C. T. particles  | 0                               | ++++                         | 4                   |
| Heparinized PRP + phospholipase C<br>0.25 mg./ml.  | 0                               | ++++                         |                     |
| Citr. PRP + ADP  | 0°                              | ++++                         | 6                   |
| Citr. PRP + C. T. particles  | 0                               | ++++                         | 4                   |
| Citr. PRP + Polybrene 0.5 mg./ml.  | 0                               | ++++                         |                     |
| Washed platelets + thrombin  | 0                               | ++++                         | 12                  |
| <i>Agglutination of Washed Platelets by Rabbit</i><br><i>Anti-Human Platelet Serum</i>                             |                                 |                              |                     |
| Serum dilution: 1:8  | ++++                            | ++++                         |                     |
| 1:32   | ++                              | ++                           |                     |
| 1:64   | 0                               | +                            |                     |
| <i>Electrophoretic Mobility</i><br>( $\mu\text{/sec.}/(\text{volt/cm.})$ )   | 1.13                            | $1.07 \pm \text{S.D. } 0.08$ | 5                   |
| <i>Isoelectric Point</i>   | pH 3.3                          | pH 3.3                       | 5                   |
| <i>Platelet Shape</i>  |                                 |                              |                     |
| Citr. or ACD PRP, 37 C.  | Discs                           | Discs                        | 13                  |
| Same plus ADP ( $1 \mu\text{M./L.}$ )  | Spiny<br>spheres                | Spiny<br>spheres             | 14                  |
| Same at 2 C.   | Spiny<br>spheres                | Spiny<br>spheres             | 13                  |
| <i>ADP Release (measured by clumping</i><br><i>activity) <math>\mu\text{M.}/10^9</math> platelets</i>              |                                 |                              | 6                   |
| ACD PRP + thrombin   | 30                              | 23–45                        |                     |
| ACD PRP + C. T. particles  | 30                              | 6–45                         |                     |
| <i>Release of Material Absorbing</i><br><i>at 260 <math>m\mu</math> (<math>\mu\text{M.}/10^9</math> platelets)</i> |                                 |                              | 6                   |
| ACD PRP + thrombin   | 50                              | 50–134                       |                     |
| ACD PRP + C. T. particles  | 53                              | 45–94                        |                     |

$10^6/\text{mm}^3$ . Since the same volume was always applied to the plates,  $11 \times 10^6$  platelets presumably contain more than  $0.5 \mu\text{g}$ . fibrinogen, or  $10^6$  platelets contain about  $0.05 \mu\text{g}$ . fibrinogen. Supernatants of suspensions containing  $16 \times 10^6$  platelets/ $\text{mm}^3$  from three other normal subjects produced easily demonstrable lines; supernatants from this concentration of the patient's platelets produced none, even when the platelets were incubated with normal citrated platelet-poor plasma for 30 minutes at room temperature prior to washing. Thus the patient's platelets contained less than  $0.03 \mu\text{g}$ . fibrinogen per  $10^6$  platelets, and normal platelets contained more. Immunofluorescent staining suggested that some of the patient's platelets were devoid of fibrinogen, while others contained a subnormal amount (Fig. 1).

For more quantitative studies, equal volumes of Fi-Test Reagent and a solution containing fibrinogen were mixed and continuously tilted on a slide, and development of grossly visible clumps was timed. When plasma or a solution of purified fibrinogen was progressively diluted, the time required for clumping increased until, at a concentration of 3 to  $4 \mu\text{g}$ . fibrinogen/ml., either a slight degree of clumping occurred in about a minute or no endpoint at all could be detected (Fig. 2). Fibrinogen concentration of an unknown solution was estimated by testing serial dilutions and assuming a fibrinogen concentration of 3 to  $4 \mu\text{g}$ . /ml. at the endpoint. The endpoint for supernatant solutions of frozen and thawed platelet suspensions of five normal individuals occurred at dilutions corresponding to 15,000, 30,000, 30,000, 40,000, and 70,000 platelets/ $\text{mm}^3$ , or about  $0.1 \mu\text{g}$ . fibrinogen per  $10^6$  platelets.

Two tests on the supernatant from thrombasthenic platelets revealed only one-seventh and one-fourth of the fibrinogen present in the control supernatant (Fig. 2), or between  $0.01$  and  $0.025 \mu\text{g}$ . fibrinogen per  $10^6$  platelets. Fibrinogen also appeared to be low when assessed by fibrin formation, since addition of thrombin to supernatants prepared from the patient's platelets ( $2.2 \times 10^6/$

Table 1.—(Continued)

|  | Patient<br>N. Y. H. =<br>793146 | Normal<br>Values | Method<br>Reference |
|--|---------------------------------|------------------|---------------------|
| <i>Serotonin: Studies with C<sup>14</sup>-Labeled Material</i> |                                 |                  |                     |
| Uptake ( $0.6 \mu\text{g}$ . added/ml. ACD PRP)                | 93%                             | 95%              | 6                   |
| Release: ACD PRP + thrombin                                    | 85%                             | 88%              |                     |
| ACD PRP + C. T. particles                                      | 63%                             | 60%              |                     |
| <i>Platelet Granules Visualized with Auramine</i>              |                                 |                  |                     |
| Citr. PRP  | Bright                          | Bright           | 15                  |
| Citr. PRP + thrombin   | Lost                            | Lost             |                     |
| <i>Acid Phosphatase (Bodansky Units)</i>                       |                                 |                  |                     |
| Serum from platelet-poor plasma                                | 0.16                            | 0.1              | 16                  |
| Serum from whole blood   | 0.40                            | 0.25             |                     |
| <i>Platelet Factor 1 (Plasma Factor V)</i>                     |                                 |                  |                     |
| Washed platelets, $100,000/\text{mm}^3$                        | 55 sec.                         | 56 sec.          | 17                  |
| 1:10 normal plasma   |                                 | 33 sec.          |                     |
| 1:40 normal plasma   |                                 | 54 sec.          |                     |
| Blank  |                                 | 250 sec.         |                     |

\*Not corrected by addition of normal plasma.

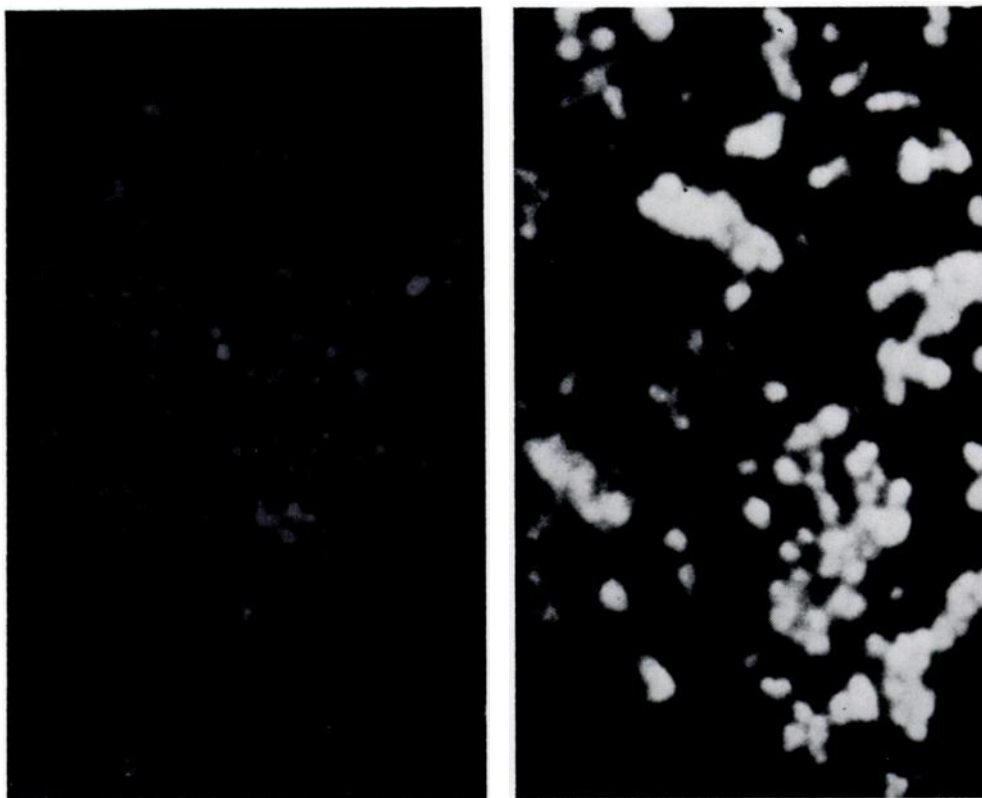


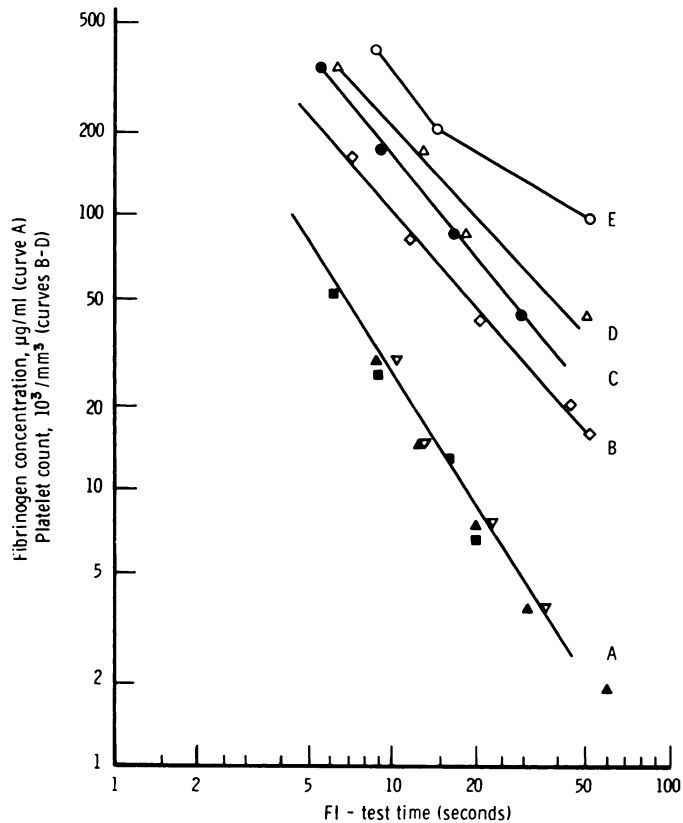
Fig. 1.—Washed platelets of (left) patient with thrombasthenia and (right) normal control after staining with fluorescein-labeled antiserum to fibrinogen.

mm.<sup>3</sup>) failed to produce microscopic fibrin needles, many of which were seen with supernatant from an equal number of normal platelets.

The endpoint for Fi-Tests on *unfrozen* suspensions of washed normal platelets occurred at about the same platelet dilution as was used for testing the supernatants. The reaction was attributed to platelet fibrinogen since control Fi-Test Reagent failed to clump either dilute or concentrated platelet suspensions. The endpoint with use of unfrozen suspensions of the patient's platelets occurred at twice the platelet concentration of the control on one occasion, but in three other tests it occurred at about the same concentration of platelets as in the control (Fig. 2). The endpoint was the same when the platelets from normal individuals or the thrombasthenic patient were tested after three, four, or five washes. The wash fluid was not tested.

#### DISCUSSION

The platelets of the thrombasthenic patient adhered normally to exposed connective tissue fibers in the mesentery,<sup>6</sup> as other investigators have reported with purified collagen,<sup>18</sup> but did not adhere to or spread on glass slides. These results support the earlier suggestion<sup>6</sup> that different mechanisms are respon-



**Fig. 2.**—Representative experiment showing double logarithmic plot of the relationship between the time required to reach the endpoint in the Fi-Test and fibrinogen concentration (curve A) or platelet count (curves B-E). Curve A represents results on purified fibrinogen (■—■), normal plasma (▽—▽), and patient's plasma (▲—▲). Curve B: supernatants of frozen and thawed suspensions of normal platelets. Curve C: suspensions of patient's intact platelets. Curve D: suspensions of normal intact platelets. Curve E: supernatant of frozen and thawed suspensions of patient's platelets.

sible for platelet adhesion to collagen and glass. Furthermore, platelets in the patient's whole citrated blood were not retained by a column of glass beads. Such retention, probably resulting from platelet adhesion to glass and aggregation by erythrocyte or platelet ADP,<sup>11,19</sup> has not been observed in other thrombasthenic patients.<sup>11,20</sup>

Although the patient's platelets did not aggregate when exposed to thrombin or connective tissue particles, their response to these substances was otherwise normal. Thrombin caused a normal decrease in platelet ATP and loss of auramine staining; clotting liberated acid phosphatase, while both thrombin and connective tissue particles released serotonin as well as ADP and other materials absorbing at 260 m $\mu$ . Jackson and co-workers<sup>21</sup> noted that thrombin caused normal consumption of platelet adenosine triphosphate (ATP) and release of

serotonin and potassium in thrombasthenia, and Inceman et al.<sup>22</sup> observed normal morphologic changes in isolated platelets of such subjects during clotting.

The patient's platelets did not aggregate with ADP. Similar results have been reported by others.<sup>20-22</sup> This deficiency was not corrected by addition of magnesium, although our team and others noted that clot retraction was improved by the addition of 0.01 M.<sup>3,23</sup> Hardisty et al.<sup>20</sup> observed that platelets from thrombasthenic patients failed to aggregate with epinephrine or serotonin, and we noted that other agents possibly acting through ADP were also ineffective (Table 1). Antiplatelet serum produced normal platelet agglutination in our case. The electrophoretic mobility and isoelectric point of the platelets were also normal. The platelets had a normal disc shape in ACD or citrated PRP at 37 C.<sup>13</sup> and underwent the usual morphologic changes when subjected to ADP<sup>14</sup> or chilling.<sup>13</sup> The abnormality in the thrombasthenic platelets' response to ADP is evidently not in the first step associated with shape<sup>14</sup> and volume change,<sup>24</sup> but rather in a later step that can be blocked by EDTA<sup>14</sup> or tosyl arginine methylester (TAME).<sup>25</sup>

Most earlier investigators found that thrombasthenic platelets promoted normal prothrombin consumption and activity in the TGT.<sup>1</sup> However, Hardisty et al.<sup>20</sup> and our own group noted abnormal prothrombin consumption. Deficient response of thrombasthenic platelets to kaolin<sup>26,27</sup> constitutes further evidence that such platelets are unable to contribute normal platelet Factor 3 activity during coagulation. Kaolin also failed to release ADP from thrombasthenic platelets.<sup>20,26</sup>

The patient had normal levels of ATP and glyceraldehyde-3-phosphate-dehydrogenase in the platelets. Values were low in most of the thrombasthenic patients studied by Löhr, Waller, and Gross,<sup>23</sup> but normal in the majority of such patients subsequently studied by others.<sup>1</sup> Abnormalities of other platelet enzymes have also been reported.<sup>1</sup> The Factor 5 activity of our patient's platelets was normal; similar results were noted by Larrieu et al.<sup>3</sup>

The fibrinogen concentration in the supernatant of normal frozen and thawed platelets was about 0.1  $\mu\text{g.}/10^6$  platelets when estimated by the Fi-Test, and over 0.03  $\mu\text{g.}/10^6$  platelets according to immunoelectrophoresis. Similar values were obtained by actual measurement of clottable protein in supernatant: Johnson et al.<sup>28</sup> found 0.18  $\mu\text{g.}/10^6$  platelets in one normal human; Grette<sup>29</sup> found an average of 0.04  $\mu\text{g.}/10^6$  in two experiments on pig platelets; and we found 0.1  $\mu\text{g.}/10^6$  in one normal individual.

In contrast, supernatants of the patient's platelets contained 0.01 to 0.03  $\mu\text{g.}/10^6$  platelets. These results, briefly presented earlier,<sup>7</sup> have been confirmed.<sup>18</sup> Jackson et al.<sup>21</sup> independently observed that supernatants of frozen and thawed platelets from a patient with thrombasthenia failed to clot with thrombin. Two earlier reports of patients with a low concentration of clottable protein, presumably fibrinogen, in platelet extracts have been largely overlooked. One patient had thrombasthenia;<sup>30</sup> the other had 2 to 3+ clot retraction with a lifelong history of bleeding, prolonged bleeding time, positive tourniquet test, and short prothrombin consumption time.<sup>28</sup> Castaldi and

Caen,<sup>31</sup> using the Fi-Test, found fibrinogen in the supernatant of homogenized centrifuged normal, but not thrombasthenic, platelets.

The equal Fi-Test activity of unwashed platelet suspensions and supernatants which we noted, and the presence of fibrinogen in the wash solution after eight washes,<sup>31</sup> suggest that this protein is present on the surface of normal platelets, yet Nachman, Marcus, and Zucker-Franklin<sup>32</sup> failed to detect it in the membrane fraction of homogenized platelets. Since the location of fibrinogen in normal platelets is not known, it is impossible to interpret the observation that thrombasthenic platelets have low fibrinogen when wash fluids<sup>31</sup> or supernatants are tested, but normal fibrinogen when sediments<sup>31</sup> or unwashed platelets are used. Nachman's results,<sup>33</sup> using platelets in which virtually all of the protein was solubilized by ultrasound (personal communication), provide the most convincing evidence that thrombasthenic platelets have a subnormal total quantity of fibrinogen rather than an abnormal distribution. He found that the platelets of our 10-year-old patient contained about 10 per cent of the normal amount of fibrinogen.

Although platelet fibrinogen may be involved in reactions to thrombin,<sup>34</sup> and exogenous fibrinogen may be important for ADP-induced platelet aggregation,<sup>35</sup> the relationship of the low platelet fibrinogen to the functional deficiencies of thrombasthenic platelets is not clear. It may explain the poor adhesion to glass<sup>36,37</sup> but probably does not account for the absence of ADP-induced aggregation.<sup>38</sup>

#### SUMMARY

The platelets of a patient with congenital thrombasthenia were not aggregated by ADP, thrombin, connective tissue particles, Polybrene, or phospholipase C, and did not adhere to glass as measured either on a glass slide or by retention in a glass-bead column. Clot retraction was markedly diminished. Raising the magnesium level partially corrected clot retraction but did not restore ADP-induced clumping. The platelets were less able to promote prothrombin consumption. Fibrinogen concentration in the supernatant of frozen and thawed platelets was low, but surface fibrinogen appeared to be normal.

The thrombasthenic platelets were normal in the following respects: concentration of ATP and glyceraldehyde-3-phosphate-dehydrogenase; adhesion to connective tissue fibers; aggregation by antiplatelet serum; microelectrophoretic mobility; isoelectric point; disc shape of platelets at 37 C.; ability of platelets to change shape with ADP or cold; decrease in ATP concentration and auramine staining of granules by thrombin; release of serotonin, ADP, and other materials absorbing at 260  $m\mu$ . by thrombin or connective tissue particles; liberation of acid phosphatase during blood clotting; and platelet Factor 5 activity.

It is concluded that responses of thrombasthenic platelets to thrombin and connective tissue particles are normal except that the liberated ADP fails to cause aggregation. The first stage of the reaction to ADP, transformation from disc to spiny sphere, is normal. Still to be determined at the molecular level is the cause(s) of failure of clot retraction and ADP-induced aggregation and the



relationship of these defects to the low fibrinogen concentration of platelet extracts.

#### SUMMARIO IN INTERLINGUA

Le plachettas de un patiente con congenite thrombasthenia non esseva aggregate per diphosphato de adenosina, per thrombina, per particulas de tissu conjunctive, per Polybreno, o per phospholipase C, e illos non adhereva a vitro a judicar per mesurationes super laminas de vitro o per le observation del retention de illos in un columna de perlas de vitro. Le retraction del coagulo esseva marcatamente reducite. Per augmentar le nivello de magnesium il esseva possibile corrigere in parte le retraction del coagulo, sed iste mesura non restaurava le aggregatura inducite per diphosphato de adenosina. Le plachettas esseva minus capace a promover le consumption de prothrombina. Le concentration de fibrinogeno in le liquido supernatante de congelate e re-disgelate plachettas esseva basse, sed le fibrinogeno al superficie pareva esser normal.

Iste plachettas thrombasthenic esseva normal in le sequente respectos: Concentration de triphosphato de adenosina e de glyceraldehyda-3-phosphatodehydrogenase; adhesion a fibras de tissu conjunctive; aggregation per sero anti plachettas; mobilitate microelectrophoretic; puncto isoelectric; forma discoide a 37 C; capacitate de alterar le conformation sub le influenza de diphosphato de adenosina o de frigiditate; declino del concentration de triphosphato de adenosina e del tincturation a auramina del granulos sub le influenza de thrombina; liberation de serotonina, diphosphato de adenosina, e altere materiales a absorption a 260 m $\mu$  per thrombina o particulas de tissu conjunctive; liberation de phosphatase acide durante coagulation sanguinee; e activitate de Factor plachettal V.

Es concludite que responsas de plachettas thrombasthenic a thrombina e particulas de tissu conjunctive es normal con le exception que le liberate diphosphato de adenosina non causa aggregation. Le prime stadio del reaction a diphosphato de adenosina, i.e., le transformation ab discos ad sphas spinose, es normal. Remane a determinar al nivello molecular le causa o causas del defecto in le retraction del coagulo e in le aggregation inducite per diphosphato de adenosina e le relation inter iste defectos e le basse concentration de fibrinogeno in extractos del plachettas.

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