In Vitro Detection of Platelet Antibody in Patients with Idiopathic Thrombocytopenic Purpura and Systemic Lupus Erythematosus

By Simon Karpatin and Gregory W. Siskind

The existence of human platelet antibodies and their deleterious effect on platelet survival has been well established. Incontrovertible evidence for the existence of heterologous antibodies as well as autologous drug-induced autoantibodies against human platelets has existed in the literature for more than 20 years. More recent work on human platelet isoantigens and isoantibodies has been reported from several laboratories. However, evidence for the existence of autoantibodies in idiopathic thrombocytopenic purpura (ITP) has not been unanimously accepted.

The unique experiments of Harrington and co-workers in 1951 provided incontrovertible evidence for the existence of a humoral factor in the plasma of patients with ITP, capable of bringing about a precipitous fall in platelet count when infused into normal subjects. These workers also demonstrated the existence of platelet agglutinins in the sera of 66 per cent of patients with ITP. Further evidence for the existence of a humoral factor in patients with ITP may be obtained from the clinical observations of neonatal purpura in infants of mothers with ITP as well as the reports of other investigators on the existence of a platelet agglutinin in ITP patients. This agglutinin has been shown to be present in the gamma globulin fraction of serum.

Unfortunately, in vitro detection of platelet agglutinins has not been reproducible in all laboratories, and platelet agglutinin work with sera has been challenged because of the presence of thrombin or thrombin-generating agents in these sera which are capable of agglutinating platelets. Thus, although the presence of a humoral antiplatelet factor in patients with ITP is unquestioned, the characterization of this factor as an antibody has not been rigorously established. The antiglobulin consumption test has also been used to detect antiplatelet activity in some cases of ITP. However, this complex technic has been criticized because of its tendency to give false positive re-

From the Department of Medicine, New York University School of Medicine, New York, N. Y.

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Simon Karpatin, M.D.: Assistant Professor of Medicine; Career Scientist of the Health Research Council of the City of New York, I-459. Gregory W. Siskind, M.D.: Assistant Professor of Medicine; Career Scientist of the Health Research Council of the City of New York, I-464.
suits with normal controls. Standard immunologic procedures not dependent upon agglutination technics thus far have yielded negative results.

The present communication will present evidence for the in vitro detection of an antiplatelet factor under conditions which rule out the possibility of thrombin or thrombin-generating agents being responsible. This antiplatelet factor is detectable by two simple tests which employ a globulin fraction of the patient's sera and normal human platelets.

1) A macroscopic platelet agglutinin technic employing Dextran to increase the agglutination sensitivity.

2) A more sensitive platelet-injury technic wherein the ability of platelets to make their phospholipid-platelet-factor 3 available to the coagulation cascade is assayed.

Evidence will be presented for the characterization of this antiplatelet factor as an IgG immunoglobulin.

MATERIALS AND METHODS

Human platelet-rich plasma as well as washed platelets was collected and handled as described previously. Plastic equipment was used at all times. Platelet-rich plasma was employed for the Platelet Factor 3 test (PF-3) and washed pooled platelets suspended in human Ringer solution containing 0.1mM EDTA was utilized for the Dextran-Platelet Agglutination Test.

The test material for platelet antibody activity was prepared from serum obtained from shed blood or by addition of sufficient CaCl₂ to coagulate blood collected in ACD solution or EDTA. A globulin fraction was prepared by (NH₄)₂SO₄ precipitation upon addition of an equal volume of cold-saturated (NH₄)₂SO₄. The globulin fraction was collected by centrifugation and dissolved in its original serum volume of phosphate-buffered saline. This globulin solution was then dialyzed extensively against phosphate-buffered saline (PBS = 0.1M potassium phosphate buffer, 0.15M NaCl, pH 7.4).

Dextran-Platelet Agglutination Test

All reagents contained 0.1mM EDTA. This was necessary to prevent nonspecific ADP clumping.* The following were added to a 5 ml. plastic tube and incubated at 37 C. for 180 minutes: 0.25 ml washed human platelets suspended in human Ringer solution at a packed platelet concentration of 5 per cent (v/v), 0.1 ml. of 6 per cent Dextran (molecular weight approximately 70,000) in 0.9 per cent saline, and 0.25 ml. of a globulin fraction of the serum to be tested in PBS. Tubes were checked for macroscopic agglutination at 15-minute intervals for 3 hours. Two control tubes were always run simultaneously with the test samples. These controls consisted of 1) addition of globulin fraction of normal human serum instead of test globulin fraction to the above reagents and 2) addition of human Ringer solution containing 0.1mM EDTA (human Ringer-0.1mM EDTA) instead of test globulin fraction to the above reagents. If either control exhibited agglutination over the period of observation the results of the incubation were discarded. Washed platelets could be stored for up to 24 hours at 4 C. and still utilized without clumping in either control. Sera showing agglutination upon initial testing were titered by diluting the globulin fraction in human Ringer-0.1mM EDTA. Values for titer potency are given in terms of maximal dilution of globulin capable of giving positive results (macroscopic agglutination within 3 hours) in the test system described above.

* Larger concentrations of EDTA, in the order of 1-3mM were noted to inhibit the rabbit anti-human platelet antibody agglutination. This was not a result of inhibition of complement activity since decomplemented sera was capable of causing agglutination of platelets.
Platelet Factor 3 Test

This test is a modification of the Hardisty procedure. The following modifications were employed: 1) elimination of kaolin from the procedure thus making antibody the sole agent causing platelet injury, and 2) measurement of phospholipid "release" at two stages (activation of factor VIII as well as conversion of prothrombin to thrombin by activated X) rather than the one stage employed in the Russel Viper venom (or product I) technics.

The test is carried out as follows: To 0.1 ml. of platelet-rich plasma in a plastic tube (collected as 1 part ACD to 5 parts blood and stored at 4 C. until use) was added 0.1 ml. of "contact product" in normal saline prepared by described by Nossel, and 0.1 ml. of a globulin fraction of the serum to be tested. (Briefly, "contact product" was prepared by adding fresh platelet-poor plasma (ACD) to celite (1 ml. plasma per 20 mgm. celite). This suspension is shaken by inversion every 2 minutes at 37 C. for 10 minutes. The celite (adsorbed "contact product") is harvested by centrifugation and resuspended in distilled water (original plasma volume). The celite is then removed and washed 5 times with distilled water. The celite is then suspended in 10 per cent NaCl using one half the original plasma volume. This suspension is then kept at 37 C. for 10 minutes, mixing by inversion every 2 minutes. The celite is then discarded and the supernatant containing the eluted "Contact Product" dialyzed for 24 hours against 0.85 per cent NaCl at 4 C. (1:75). This mixture was incubated at 37 C. for 5 minutes followed by the addition of 0.1 ml. of 0.025 M CaCl₂ to initiate coagulation. The fibrin end point was observed and was recorded in seconds. All tests were performed in duplicate and agreed within 3 or 4 seconds. Globulin fractions of normal sera gave a clotting time of 85–95 seconds. Results were considered positive if the clotting time was shortened by 10 or more seconds as compared with a simultaneously run control on at least two separate determinations carried out on different days employing a minimum of two different platelet-rich plasmas. Samples which gave positive results were titered employing the globulin fraction of normal serum as diluent. This was necessary to keep the concentration of globulin and of clotting factors present in the globulin fraction constant and resulted in a higher sensitivity for the test system. Addition of globulin fraction prepared from normal sera or from sera from ITP patients to platelet-poor plasma in the test system did not shorten the clotting time.

Prothrombin was assayed by the one-stage method of Pechet.

G-200 Sephadex gel filtration was utilized to obtain an approximate estimate for the apparent molecular weight of the antiplatelet factor employing the technic of Andrews. A column 8 x 1.5 cm was equilibrated at 4 C. with isotonic saline. One ml. of globulin fraction (25–30 mgm protein) was dialyzed against isotonic saline and placed on the column. One ml samples were collected, the 280 mₐ absorbance of odd numbered tubes determined and the even numbered tubes assayed for antiplatelet activity employing the PF-3 test. The marker proteins employed for the sephadex column calibration were: beef liver catalase, 250,000, goat gamma globulin, 156,000, human hemoglobin, 66,700, horse heart cytochrome-C, 12,500.

Immunoelectrophoresis was performed on agar gel slides in veronal buffer, pH 8.6, ionic strength 0.1, at 30 volts for two hours at room temperature. Antisera was then added to the central trough and the plates held overnight at 4 C. in a humidified chamber to develop the immunoelectrophoresis pattern.

Patient material

Sera from 26 patients with ITP were obtained from the Bellevue Hospital wards and Hematology Clinic, from University Hospital, and from referrals by several physicians in the community. All patients designated with the diagnosis of ITP had severe thrombocytopenia (platelet counts of <10,000 to 90,000) without leukopenia, anemia, or splenomegaly, negative S.L.E. preparations and negative or nonspecific results with immuno-fluorescent studies for antinuclear antibodies. All patients had either two or more episodes

*These studies were kindly performed by Dr. Naomi Rothfield, director of the New York University—Bellevue Hospital S.L.E. clinic.
of acute thrombocytopenia or chronic thrombocytopenia. As a comparison, sera from 33 unselected patients with systemic lupus erythematosus (S.L.E.) were obtained from the S.L.E. clinic at Bellevue Hospital through the courtesy of Dr. Naomi Rothfield. All had positive S.L.E. preparations and positive tests for antinuclear antibodies on at least one occasion. Two of these 33 patients had low platelet counts (120 and 156,000 per cubic mm). Twenty-six sera from patients with thrombocytopenia not due to ITP or SLE were obtained from patients in Bellevue Hospital or University Hospital. Twenty-five normal control sera were obtained from healthy laboratory personnel or blood donors from The New York Blood Center. None of the SLE patients studied had a history of previous blood transfusion. Of the ITP patients studied, 4 had histories of previous whole blood transfusion. None of these received more than 4 units of blood. Of the 4 thrombocytopenic controls with positive Dextran agglutination tests, one patient (H.A.) gave a history of having received numerous blood transfusions in the past. It has been pointed out by Shulman that "even after ten transfusions the frequency of isoimmunization detectable by in vitro tests is less than 5 per cent.”

All chemicals were of reagent grade. Purified human thrombin was a gift of Dr. Kent Miller. EDTA, Tris buffer, catalase and cytochrome-C were obtained from Sigma Chemical Co., St. Louis, Mo. Goat gamma-globulin was obtained from Immunology Inc., Lombard, Illinois. Dextran-70 was obtained from Travenol Laboratories, Morton Grove, Illinois. Specific rabbit antisera against IgG, IgA, IgM and IgD were obtained from Hoechst Pharmaceutical Co., Kansas City, Missouri.

Per Cent Large Platelets

This is the per cent of large platelets per total platelets counted on peripheral smear. Peripheral smears were made from blood collected in EDTA and stained with McNeil tetrachrome stain. A minimum of 50 large platelets were counted under 1000 X magnification. Large platelets were defined as those having a diameter greater than 2.5 μ. Twenty-seven control smears were obtained from relatively healthy patients with normal white blood cell, hematocrit, and platelet levels and without systemic disease, who were admitted to University Hospital for minor elective surgical procedures (plastic surgery, inguinal hernia repair, cataract removal, etc.). A control value of 11 ± 3 (S. D.) with a range of 7 to 16 was obtained. Values of 17 per cent or more for the per cent large platelets (2 standard deviations from the control value, P < .05) were regarded as significantly increased.

RESULTS

Demonstration of Antiplatelet Factor

Residual prothrombin activity in globulin fractions. In order to determine whether the antiplatelet factor could be merely residual prothrombin activity present in the globulin fraction which is converted to thrombin by platelets, measurements† of residual prothrombin activity were compared with assay titers of various samples. As can be seen in Table 1 no correlation exists between prothrombin activity and the antiplatelet factor activity as measured by the PF-3 or Dextran agglutination assays. Thus the antiplatelet factor being studied here is not prothrombin.

Addition of thrombin to control sera. The possibility remained that thrombin might be present in the globulin fractions prepared for assay of antiplatelet factor activity. Human thrombin, freshly prepared and assayed, was

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* Large platelets have been associated with young platelets, see discussion.
† These measurements were kindly performed by Dr. Margaret Howell-Karpatin of the Department of Pediatrics, NYU Medical School.
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Table 1.—Residual Prothrombin Remaining in Globulin Fractions as Compared with Dextran-Agglutinin and PF-3 Titers

<table>
<thead>
<tr>
<th>Patient</th>
<th>% Residual Prothrombin</th>
<th>Dextran-Agglutinin Titer</th>
<th>PF-3 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK</td>
<td>114</td>
<td>0</td>
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<tr>
<td>KS</td>
<td>91</td>
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<td>0</td>
</tr>
<tr>
<td>AP</td>
<td>59</td>
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<td>20</td>
</tr>
<tr>
<td>YH</td>
<td>15</td>
<td>19.2</td>
<td>68</td>
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<tr>
<td>AF</td>
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<td>32</td>
</tr>
<tr>
<td>CV</td>
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<td>0</td>
</tr>
<tr>
<td>RD</td>
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<td>16</td>
</tr>
<tr>
<td>BE</td>
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<td>32</td>
</tr>
<tr>
<td>CC</td>
<td>&lt;1</td>
<td>2.4</td>
<td>22</td>
</tr>
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</table>

* Refers to the per cent prothrombin remaining in the globulin fraction of serum. Pooled normal plasma from healthy donors was used as 100 per cent reference control, (see methods).

† Results are expressed as the highest dilution of globulin fraction giving a positive test. "0" indicates a negative test using undiluted globulin.

Table 2.—Effect of Preparation of Globulin Fraction Upon Thrombin Activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation times (minutes at 37 C.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>120</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
</tr>
<tr>
<td>Serum</td>
<td>4+</td>
</tr>
<tr>
<td>Globulin</td>
<td>0</td>
</tr>
<tr>
<td>Serum + 6.7T †</td>
<td>4+</td>
</tr>
<tr>
<td>Serum + 17.5T</td>
<td>4+</td>
</tr>
<tr>
<td>Globulin prepared from Serum + 6.7T</td>
<td>0</td>
</tr>
<tr>
<td>Globulin prepared from Serum + 17.5T</td>
<td>0</td>
</tr>
</tbody>
</table>

* A simple agglutination test was employed, wherein PBS was substituted for dextran, see dextran-agglutination test, methods.

† Amount of thrombin in N.I.H. units/ml.

added to normal sera at concentrations of 6.7 and 17.5 N.I.H. units/ml. Globulin fractions were then prepared as usual (see methods section) and assayed in the washed platelet agglutinin test. As can be noted from Table 2, thrombin was either removed or inactivated during the preparation of the globulin fractions. The washed platelet agglutinin system (no Dextran present) could detect 1 milli-unit/ml thrombin but failed to detect 0.9 milli-units/ml. The sera obtained from platelet-poor plasma when serially diluted caused agglutination of platelets at a final dilution of 1:80 and caused no agglutination at 1:90. Thus human serum is equivalent to 0.08 units/ml of a standard thrombin solution with respect to its ability to agglutinate platelets during 180 minutes of incubation at 37 C. (Table 3).

Sensitivity of technics. The dextran-agglutination technic was found to be three times more sensitive than a routine agglutination technic (substituting PBS solution for dextran-saline in the reaction mixture) when employing a globulin fraction of rabbit antihuman platelet antibody. A similar threefold
Table 3.—Effect of Serum and Thrombin on Platelet Agglutination *

<table>
<thead>
<tr>
<th>Sample (dilution)</th>
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<th>180</th>
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<tr>
<td>Saline</td>
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<td>0</td>
</tr>
<tr>
<td>Serum A (1:80)</td>
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<td>+</td>
</tr>
<tr>
<td>Serum A (1:90)</td>
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<td>0</td>
</tr>
<tr>
<td>Serum B (1:80)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Serum B (1:90)</td>
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<td>0</td>
</tr>
<tr>
<td>Serum C (1:80)</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Serum C (1:90)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thrombin Concentration (Milli-units/ml.)</th>
<th>0.9</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

* Same test system employed as in Table 2.

increase in sensitivity was obtained in one patient who had a sufficiently high agglutinin titer to be detectable by the simple agglutination technic. Using a globulin fraction of a rabbit antihuman platelet antiserum as a standard, the PF-3 test was four times more sensitive than the dextran agglutination technic and 12 times more sensitive than the simple agglutination technic. With human material the PF-3 test appeared to be even more sensitive as compared with the dextran platelet agglutination assay. Thus, with ITP patients, the average PF-3 test titer was 6 times greater than the average titer of their dextran-agglutination tests. In a group of 33 S.L.E. patients, the average titer of the PF-3 test was 10 times greater than the average titer of the Dextran-platelet agglutination test.

Reproducibility of technics. The dextran-agglutination test and the PF-3 test were reasonably reproducible from day to day employing different washed platelet pools (see Tables 4A and 4B). It should be emphasized that the data presented are completely unselected, all our data being offered in the tables.

Variation with time. Samples from S.L.E. patients were obtained at varying times during their illness. As can be seen in Table 5, the dextran-agglutination titers of a given patient’s serum remained fairly constant over a 3 to 4 month period. During the same period of observation some variation in the PF-3 Test titer was observed.

Antiplatelet factor assays of normal subjects, ITP patients, SLE patients and patients with thrombocytopenia secondary to other hematologic disorders. Twenty-six patients with active ITP were tested (Fig. 1). Dextran-agglutination titers varied from 0 to 18.0 with an average of 4.9 for those patients showing positive reactions. Positive dextran-agglutinin titers were found in 65 per cent of patients. PF-3 titers varied from 0 to 68. Positive PF-3 titers averaged 27 and were present in 73 per cent of patients tested. The correlation coefficient for both tests in this group of patients was highly significant, \( r = 0.69, P < 0.05 \). Four out of 26 patients had negative dextran-agglutinin and PF-3 tests. Thus, 85 per cent of patients had either a positive agglutinin or a positive PF-3 test. Five out of 26 patients had negative
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Table 4A.—Reproducibility of Dextran-Agglutination and PF-3 Tests in Patients with ITP *

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dextran-Agglutinin Titer</th>
<th>PF-3 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>HE</td>
<td>19.2</td>
<td>16.8</td>
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<tr>
<td>AM</td>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td>RI</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>ST</td>
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<td>2.4</td>
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<tr>
<td>LA</td>
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<td>2.4</td>
</tr>
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<td>OS</td>
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<td>0</td>
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<tr>
<td>DE</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

* Results of repeated tests using the same globulin sample and different platelet preparations. Data are unselected.

† Titers are expressed as the highest dilution of the globulin preparation giving a positive result. "0" indicates a negative result with undiluted sample.

Dextran-agglutinin tests, but positive PF-3 tests. Of these five patients, the highest PF-3 titer was 16. Three out of 26 patients had negative PF-3 tests and positive dextran-agglutinin tests, the highest titer being 4.8.

Systemic Lupus Erythematosus Patients. Thirty-three patients with S.L.E. were tested (Fig. 1). Only two of these 33 had thrombocytopenia. Dextran-agglutinin titers varied from 0 to 9.6 with an average of 3.9 for the positive patients. Positive dextran-agglutinin tests were found in 67 per cent of patients. PF-3 test titers varied from 0 to 54 with an average of 31 for the positive patients. Positive PF-3 titers were found in 85 per cent of patients. The correlation coefficient for both tests was \( r = 0.33, P < 0.05 \). In four out of 33 patients both dextran-agglutinin and PF-3 tests were negative. Thus, 88 per cent of patients had either a positive agglutinin or PF-3 test. Seven patients had negative dextran-agglutinin and positive PF-3 tests and two patients had negative PF-3 tests and positive dextran-agglutinin tests. The two patients with thrombocytopenia had positive dextran-agglutinin titers of 9.6 and 3.0, and positive PF-3 test titers of 52 and 24.

The per cent "large platelets"* was also investigated in all S.L.E. patients with normal platelet counts to see whether any correlation could be ob-

*Large platelets have been associated with young platelets, see discussion.
Table 4B.—Reproducibility of Dextran-Agglutination and PF-3 Tests in Patients with SLE *

<table>
<thead>
<tr>
<th>Patients</th>
<th>Dextran-Agglutinin Titer</th>
<th>PF-3 Titer</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>MC</td>
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<tr>
<td>CA</td>
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<tr>
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<td>2.4</td>
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<tr>
<td>LE</td>
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<td>2.4</td>
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<tr>
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<tr>
<td>RI</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>TO</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>AD</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>AD(18)</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>KO</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>CAS</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>PE</td>
<td>2.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Results of repeated tests using the same globulin sample and different platelet preparations. Data are unselected.
† Number following patient's initials indicates a second serum and the time interval in weeks between the two samples.
‡ Titers are expressed as the highest dilution of the globulin preparation which gave a positive result. "0" indicates a negative result with undiluted sample.

...tained with the number of large platelets and the titer of dextran-agglutinin or PF-3 test. The average per cent large platelets in 27 control patients was 11 ± 3, with a range of 7 to 16. The entire group of 33 S.L.E. patients studied averaged 24 per cent large platelets. The two thrombocytopenic patients were both 25 per cent. Of the 33 patients studied 75 per cent had an increased number of large platelets. The three patients with both negative dextran-agglutinin and PF-3 tests averaged 13 per cent large platelets. These...
### Table 5.—Variation in Antiplatelet Factor(s) Activity with Time *

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dextran-Agglutinin Titer</th>
<th>Time Interval Between Serum Samples (weeks)</th>
<th>PF-3 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA</td>
<td>2.4, 2.4</td>
<td>2</td>
<td>28, 40</td>
</tr>
<tr>
<td>MC</td>
<td>2.4, 2.4</td>
<td>10</td>
<td>28, 40</td>
</tr>
<tr>
<td>LI †</td>
<td>2.4, 0</td>
<td>13</td>
<td>0, 36</td>
</tr>
<tr>
<td>CA</td>
<td>2.4, 2.4</td>
<td>13</td>
<td>16, 40</td>
</tr>
<tr>
<td>DAV</td>
<td>2.4, 2.4, 2.4</td>
<td>14, 29</td>
<td>132, 40, 40</td>
</tr>
<tr>
<td>FO</td>
<td>0, 0</td>
<td>14</td>
<td>132, 32</td>
</tr>
<tr>
<td>GE</td>
<td>4.8, 4.8</td>
<td>16</td>
<td>40, 40</td>
</tr>
<tr>
<td>KA</td>
<td>0, 0</td>
<td>16</td>
<td>16, 16</td>
</tr>
<tr>
<td>CAR</td>
<td>2.4, 0</td>
<td>17</td>
<td>20, 24</td>
</tr>
<tr>
<td>AD</td>
<td>4.8, 2.4</td>
<td>18</td>
<td>24, 24</td>
</tr>
<tr>
<td>CAR</td>
<td>2.4, 2.4</td>
<td>18</td>
<td>24, 24</td>
</tr>
<tr>
<td>IM</td>
<td>0, 2.4</td>
<td>19</td>
<td>0, 40</td>
</tr>
<tr>
<td>DAM</td>
<td>2.4, 2.4</td>
<td>20</td>
<td>0, 0</td>
</tr>
<tr>
<td>RO</td>
<td>0, 0</td>
<td>31</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

* All data are unselected. Titers reported are the highest dilution of globulin fraction showing a positive result. "0" indicates a negative reaction with undiluted globulin.

† Patient with ITP, all other patients had SLE.

---

**Fig. 1.—Dot scattergram of antiplatelet factor titers.** The maximum positive titer of antiplatelet factor obtained from globulin fractions of sera from normal subjects and patients with ITP, SLE and thrombocytopenic states are depicted for both the Dextran-agglutinin as well as the Platelet Factor 3 test (see methods for details).
Table 6.—Thrombocytopenic Control Patients *

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Platelet Count per cu. mm.</th>
<th>Dextran-Agglutinin Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laennec's Cirrhosis &amp; Hepatosplenomegaly</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY</td>
<td>118,000</td>
<td>0</td>
</tr>
<tr>
<td>FO</td>
<td>53,000</td>
<td>0</td>
</tr>
<tr>
<td>HA</td>
<td>70,000</td>
<td>0</td>
</tr>
<tr>
<td>FR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LO</td>
<td>156,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Infectious Mononucleosis &amp; Hepatosplenomegaly</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td>114,000</td>
<td>0</td>
</tr>
<tr>
<td>KE</td>
<td>90,000</td>
<td>0</td>
</tr>
<tr>
<td>BI †</td>
<td>14,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Drug Reaction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FE, Orinase, presumptive</td>
<td>10,000</td>
<td>0</td>
</tr>
<tr>
<td>FA, Quinidine, proven</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>BI, Dapramine, proven folate deficiency</td>
<td>14,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Agnogenic Myeloid Metaplasia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BE</td>
<td>21,000</td>
<td>4.8</td>
</tr>
<tr>
<td>PA</td>
<td>50,000</td>
<td>0</td>
</tr>
<tr>
<td>AN</td>
<td>30,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Leukemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA, Chronic Myelocytic</td>
<td>130,000</td>
<td>0</td>
</tr>
<tr>
<td>HE, Chronic Lymphocytic</td>
<td>39,000</td>
<td>0</td>
</tr>
<tr>
<td>WI, ? Subacute Monocytic</td>
<td>3,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Thalassemia</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA, thalassemia major &amp; hepatosplenomegaly</td>
<td>52,000</td>
<td>0</td>
</tr>
<tr>
<td>BA, thalassemia intermedia &amp; splenomegaly</td>
<td>85,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Thrombotic Thrombocytopenic Purpura</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VA</td>
<td>80,000</td>
<td>0</td>
</tr>
<tr>
<td>WH</td>
<td>13,000</td>
<td>0</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME, Pernicious Anemia</td>
<td>73,000</td>
<td>2.4</td>
</tr>
<tr>
<td>RI, Malignant Hypertension &amp; Uremia</td>
<td>124,000</td>
<td>0</td>
</tr>
<tr>
<td>SE, Defibrination syndrome</td>
<td>45,000</td>
<td>0</td>
</tr>
<tr>
<td>RY, Prosthetic valve &amp; Rheumatic Heart Disease</td>
<td>30,000</td>
<td>0</td>
</tr>
<tr>
<td>HA, Auto-immune Hemolytic Anemia</td>
<td>114,000</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* All PF-3 tests were negative.
† Patient had combined Infectious Mononucleosis (positive Heterophile) plus folate deficiency secondary to Dapramine.
†† Titers are the highest dilution of globulin showing a positive test result. "0" indicates a negative test result with undiluted globulin.

Data suggest that large platelets are associated with positive antiplatelet factors as measured by agglutinin or PF-3 tests in patients with S.L.E.

Normal control patients. Twenty-five consecutive control sera obtained from healthy laboratory personnel or blood donors were negative for both dextran-agglutinin and PF-3 tests (Fig. 1).
IN VITRO DETECTION OF PLATELET ANTIBODY

Table 7.—Absorption of Antiplatelet Factor with Washed Platelets *

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dextran-Agglutinin Titer</th>
<th>PF-3 Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>TE</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>VE</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>DE</td>
<td>4.8</td>
<td>2.4</td>
</tr>
<tr>
<td>BL</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>CO</td>
<td>2.4</td>
<td>0</td>
</tr>
<tr>
<td>EB</td>
<td>2.4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Globulin fractions were assayed for antiplatelet activity in the dextran-agglutination and the PF-3 tests before and after absorption with washed human platelets for 5 minutes at 37°C. Results are reported as the highest dilution of globulin giving a positive test. "0" indicates a negative test using undiluted globulin.

† All patients had ITP.

Thrombocytopenic controls. Twenty-six patients with thrombocytopenia in the absence of ITP or SLE were studied and the results are displayed in Figure 1 and Table 6. As can be seen in Table 6 none had positive PF-3 tests. On the other hand four out of the 26 patients had platelet agglutinins demonstrable by the dextran-agglutination test.

Studies on Antiplatelet Factor

Heat stability. The antiplatelet factor present in the globulin fraction of sera from patients with ITP or SLE was found to be stable at 56°C for 30 minutes when tested with the dextran-agglutination technic as well as the PF-3 test (4 patients with SLE, 3 patients with ITP).

Absorption studies. Globulin fractions containing antiplatelet activity by the tests described above and globulin fractions from normal serum were incubated with washed human platelets, 5 per cent suspension, for 5 minutes at 37°C. The platelets were removed by centrifugation, the absorbed globulin fractions dialyzed against multiple changes of PBS and then reassayed by the dextran-agglutination and PF-3 tests. Globulin fractions of normal serum gave negative results in dextran-agglutination and PF-3 tests both before and after absorption. Six globulin fractions having antiplatelet activity showed a significant decrease in both dextran-agglutination and PF-3 titers upon absorption with washed human platelets (see Table 7).

Sephadex gel filtration. Globulin fractions from patients with high PF-3 titers were equilibrated with isotonic saline by dialysis and placed on a sephadex gel G-200 column also equilibrated with isotonic saline. The effluent from the column was assayed for antiplatelet activity in the PF-3 test. Five patients with ITP had similar patterns on elution from sephadex with an apparent molecular weight for the antiplatelet factor of 330,000 ± 40,000 (Fig. 2). Of the four patients with SLE who were tested, two had patterns similar to the ITP patients, while two had apparent molecular weights of approximately 150,000.

Characterization of antiplatelet factor. The antiplatelet activity fractions recovered from the sephadex G-200 gel filtration of globulin fractions of five
ITP patients were pooled and concentrated by ultrafiltration. This partially purified globulin fraction contained antiplatelet factor as determined by both the dextran-agglutination (positive at titer of 1:4.8) and PF-3 test (positive at titer of 1:16). Five control globulin fractions obtained from normal subjects were also filtered through sephadex G-200. Effluent from the same portion of protein profile was pooled and concentrated by ultrafiltration. This material showed no antiplatelet activity in the usual test systems.

The partially purified antiplatelet factor was examined by immunoelectrophoresis in agar gel using rabbit antiserum to human IgG, IgA, IgM, or IgD to develop the patterns. No precipitin lines were formed using anti-IgM or anti-IgD. A single precipitin line was seen with both anti-IgG and anti-IgA. The line formed with anti-IgG was of significantly greater intensity. The anti-IgG and anti-IgA preparation used gave a single line of precipitin against a globulin fraction of normal human serum with both Ouchterlony plates and immunoelectrophoresis.

Globulin fractions of rabbit anti-human IgG and rabbit antihuman IgA were diluted with an equal volume of human Ringer solution. Equal volumes of diluted antiserum and human globulin fractions containing antiplatelet activity by the dextran-agglutination test were mixed and incubated at 37 C. for one hour and then held at 4 C. overnight. The precipitates formed were removed by centrifugation at 2,700 g. for 10 minutes and the supernatant was assayed by the dextran-agglutination test. A second aliquot of the same human globulin sample was incubated with similarly diluted normal rabbit globulin. A sample of normal globulin was also incubated with diluted normal rabbit globulin to rule out nonspecific agglutination of human platelets by rabbit globulin. As can be noted from Table 8, anti-IgG antibody specifically removed the antiplatelet factor while anti-IgA antibody had no effect upon the antiplatelet activity. It should be noted that undiluted rabbit antihuman IgG
Table 8.—Effect of Treatment with Rabbit Antihuman IgG or Antihuman IgA Antibody on the Antiplatelet Factor Assayed in the Dextran-Agglutination Test*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation time (minutes) at 37 C.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
</tr>
<tr>
<td>Globulin Control</td>
<td>0</td>
</tr>
<tr>
<td>Control Globulin + NRG †</td>
<td>0</td>
</tr>
<tr>
<td>PE + NRG</td>
<td>0</td>
</tr>
<tr>
<td>PE + Anti-IgG</td>
<td>0</td>
</tr>
<tr>
<td>PE + Anti-IgA</td>
<td>0</td>
</tr>
<tr>
<td>DO + NRG</td>
<td>0</td>
</tr>
<tr>
<td>DO + Anti-IgG</td>
<td>0</td>
</tr>
<tr>
<td>DO + Anti-IgA</td>
<td>0</td>
</tr>
<tr>
<td>DA + NRG</td>
<td>0</td>
</tr>
<tr>
<td>DA + Anti-IgG</td>
<td>0</td>
</tr>
<tr>
<td>DA + Anti-IgA</td>
<td>0</td>
</tr>
<tr>
<td>PI + NRG</td>
<td>0</td>
</tr>
<tr>
<td>PI + Anti-IgG</td>
<td>0</td>
</tr>
<tr>
<td>PI + Anti-IgA</td>
<td>0</td>
</tr>
<tr>
<td>DE + NRG</td>
<td>0</td>
</tr>
<tr>
<td>DE + Anti-IgG</td>
<td>0</td>
</tr>
<tr>
<td>DE + Anti-IgA</td>
<td>0</td>
</tr>
<tr>
<td>HE + NRG</td>
<td>0</td>
</tr>
<tr>
<td>HE + Anti-IgG</td>
<td>0</td>
</tr>
<tr>
<td>HE + Anti-IgA</td>
<td>0</td>
</tr>
</tbody>
</table>

* Globulin fractions of rabbit antihuman IgA or IgG were added to human globulin fractions showing positive test results. Samples were incubated at 37 C. for 1 hour and held overnight at 4 C. Precipitates formed were removed by centrifugation and the supernate was tested by the dextran-agglutination technic. Results are reported as absence (0) or presence (+) of agglutination at varying times after onset of incubation in the dextran-agglutination system.

† NRG—Normal rabbit globulin.

†† Patient with SLE, other patients had ITP.

caused agglutination of the washed human platelet suspension. However, antihuman IgG, when diluted as described above, failed to cause agglutination.

**COMMENTS**

In vitro assay technics have been described which are capable of detecting an antiplatelet factor(s) in the serum of patients with ITP and SLE. The observations are consistent with previous reports indicating the presence of an in vivo antiplatelet factor in patients with ITP. Such observations were first reported by Harrington et al.24 and more recently confirmed by Shulman et al.30 The data reported here also confirm earlier reports of Harrington et al.25,26 with regard to the in vitro detection of a platelet agglutinin in the serum of patients with ITP.
It is obvious from the results reported that the antiplatelet factor measured by the tests described here is not residual prothrombin or thrombin. No correlation of dextran-agglutinin or PF-3 titer could be made with the presence of residual prothrombin. Furthermore, the ammonium sulfate fractionation procedure was shown to either inactivate or remove thrombin activity.

Two relatively sensitive, and reproducible procedures have thus been developed for the in vitro detection of this antiplatelet factor(s): 1) The dextran-agglutination technic takes advantage of the addition of dextran leading to the enhancement of agglutination sensitivity as reported by Levine et al. In our test, dextran enhanced the sensitivity of the simple agglutination procedure approximately threefold. The efficacy of washed pooled platelets in this procedure and the relatively constant titers obtained with different platelet pools strongly suggest that the antiplatelet factor involved is a panagglutinin. 2) Variations of the “Platelet Factor 3 Availability” test have been described by Hardisty and Hutton, Spaet and Cintron and Horowitz et al. Hardisty and Hutton employed kaolin to activate “contact product” and injure platelets so that both the entire coagulation cascade as well as the release of PF-3 from platelets was being measured. Spaet and Cintron circumvented the measurement of the beginning of the coagulation cascade by introducing activated Factor X which is required for the conversion of prothrombin to thrombin. Horowitz et al. employed the Spaet and Cintron procedure to demonstrate immunologic injury to platelets. Platelet-rich plasma when exposed to heterologous rabbit antiplatelet antibody or quinidine-requiring human antibody released PF-3 at an accelerated rate. Our PF-3 test is a modification of the Hardisty and Hutton as well as Horowitz et al. methods. Contact product is utilized rather than kaolin or activated Factor X so that enhancement of the coagulation cascade by PF-3 probably takes place at 2 loci: the activation of factor VIII as well as the conversion of prothrombin to thrombin with activated Factor X. It is conceivable that these modifications, as well as the dilution of the antiplatelet factor in normal globulin, enhance the sensitivity of the immunologic platelet injury test as manifested by the release of PF-3. No direct comparison of sensitivity between the procedure as modified here and the Spaet-Horowitz procedure was made. Horowitz et al. did not detect platelet antibodies in serum of patients with ITP employing the Spaet-Horowitz procedure, whereas we were able to detect antiplatelet activity in 73 per cent of patients with ITP and 85 per cent of patients with SLE. This procedure was approximately 25 times more sensitive than simple agglutination technics.

The antiplatelet factor(s) in patients with ITP and SLE appears to be an IgG immunoglobulin. It is stable at 56°C., absorbed by platelets and specifically removed by rabbit antihuman IgG. The apparent molecular weight of 330,000 obtained by gel filtration for the antiplatelet factors present in ITP patients remains unexplained. Possibly an aggregate of IgG is involved.

It cannot be stated with certainty whether the dextran-agglutination and the PF-3 tests are detecting the same or different antiplatelet factors. The correlation coefficient between the two tests was good in ITP patients. How-
ever, four patients had negative dextran-agglutinin tests but positive PF-3 titers of between 1:16 and 1:36. In addition, three patients had negative PF-3 tests and positive Dextran-agglutinin titers of between 1:2.4 and 1:4.8.

At least 15 per cent of patients with classic ITP had both a negative dextran-agglutinin and PF-3 test. This could represent patients with insufficient antibody for in vitro detection but sufficient antibody for the production of in vivo thrombocytopenia as demonstrated by Shulman. It might also reflect the existence of a "nonimmunologic" ITP as has been previously suggested.

Of interest was the observation of antiplatelet factor(s) in patients with SLE. This observation was not surprising in the light of the multiple auto-antibodies present in patients with this disease. However, the association of normal platelet counts with elevated titers of antiplatelet factor(s) was puzzling. It was for this reason that the percentage of "large platelets" was investigated in these patients. A strong correlation was found between the presence of antiplatelet factor and an increase in the per cent of large platelets. Biochemical and kinetic platelet survival data have recently been accumulated to demonstrate that large platelets represent young platelets. An increased per cent of young platelets suggest an increased rate of platelet turnover. It is possible that patients with SLE who have normal platelet counts in the presence of demonstrable antiplatelet factor(s) may have a compensated thrombocytolytic state, wherein production of new platelets is able to keep up with the accelerated destruction of platelets.

The negative results obtained with PF-3 tests and in most cases with the dextran-agglutination test in 26 control thrombocytopenic patients suggest that these tests are generally indicative of antiplatelet activity of an immunologic nature.

**Summary**

Two technics have been developed, which make possible the in vitro detection of platelet antibody in patients with idiopathic thrombocytopenic purpura (ITP), and systemic lupus erythematosus (SLE). The dextran-platelet agglutination technic takes advantage of the enhancement of platelet agglutination in the presence of dextran which brings about a threefold increase in simple agglutination. The Platelet-Factor-3 test measures the ability of platelet antibody to injure platelets and in so doing, make Platelet Factor 3 available to the coagulation cascade, thus accelerating the fibrin clotting time. This technic, when compared to the simple platelet agglutination test, increases the detection sensitivity of antiplatelet factor(s) twenty-fivefold.

The antiplatelet factor(s) as measured by these technics was found to be an immunoglobulin of the IgG class. The antiplatelet factor(s) was present in the sera of 85 per cent of patients with ITP and 88 per cent of patients with SLE. This antiplatelet factor(s) was absent in 25 consecutive healthy controls and in 22 out of 26 patients with thrombocytopenia not due to ITP or SLE.

Although 88 per cent of patients with SLE had demonstrable antiplatelet activity in their serum only 2 out of 33 were thrombocytopenic. These observations suggested that a compensated thrombocytolytic state was present.
in these patients. This conclusion was consistent with the good correlation between an elevated large platelet index and the presence of antiplatelet factor in these patients.

SUMMARIO IN INTERLINGUA

Ha essite disveloppate duo technicas permittente le detection in vitro de anticorpore anti thrombocytos in patientes con idiopathic purpura thrombocytopenic (IPT) e systemic lupus erythematose (SLE). Le technica del agglutination plachettal a dextrano se basa super le facto que le agglutination del thrombocytos es augmentate in le presentia de dextrano con le resultato de un triplication del simple agglutination. Le test de factor plachettal 3, del altere latere, mesura le capacitate del anticorpore anti plachettas de lesionar plachettas e, in le processso, de render disponible factor plachettal 3 pro le cascada de coagulation, con le resultato de un acceleration del tempore de coagulation fibrinose. Iste technica possede un sensibilitate pro le detection de factor (o factores) antiplachettal 25 vices plus grande que le simple test del agglutination plachettal.

Le factor (o factores) antiplachettal, mesurate per iste technicas, se revelava como immunoglobulina del classe IgG. Le factor (o factores) antiplachettal eseva presente in le seros de 85 pro cento del patientes con IPT e in 88 pro cento del patientes con SLE. Iste factor (o factores) antiplachettal eseva absentemente in 25 consecutive subjectos normal de controlo e in 22 de 26 patientes con thrombocytopenia non causate per IPT o SLE.

Ben que 88 pro cento del patientes con SLE habeva un demonstrabile activitate antiplachettal in lor sero, solo 3 in un gruppo de 33 eseva thrombocytopenic. Iste constatationes suggestiona que un compensate stato thrombocytolytic eseva presente in iste patientes. Un tal conclusion es compatibile con le facto que iste patientes es characterisate per un bon correlation inter le elevate indice de grande plachettas e le presentia de factor (o factores) antiplachettal.

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The technical assistance of Mr. Nathan Strick is gratefully acknowledged. The authors are indebted to Drs. Aaron Kellner, Fred Allen, Jr., and Carlos Ehrich of The New York Blood Center for their cooperation in the supply of human platelets. We would also like to thank Dr. Naomi Rothfield, Director of the Systemic Lupus Erythematosis Clinic at NYU-Bellevue Hospital for her help in obtaining serum from patients with SLE. Drs. Ariel Distenfeld, Ralph Nachman, Herbert Horowitz, Hymie Nossel, Aaron Marcus and Edward Amorosi are warmly thanked for their donation of sera from ITP patients. The numerous discussions and helpful suggestions of Dr. Margaret Howell-Karpatic in regard to the PF-3 test is gratefully acknowledged.

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IN VITRO DETECTION OF PLATELET ANTIBODY


In Vitro Detection of Platelet Antibody in Patients with Idiopathic Thrombocytopenic Purpura and Systemic Lupus Erythematosus

SIMON KARPATKIN and GREGORY W. SISKIND