Studies on Platelets

IX. Observations on the Properties and Mechanism of Action of a Potent Platelet Agglutinin Detected in the Serum of a Patient with Idiopathic Thrombocytopenic Purpura (with a Note on the Pathogenesis of the Disease)

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The pathogenetic mechanisms in idiopathic thrombocytopenic purpura (I.T.P.) are still matters of dispute. The coexistence of thrombocytopenia in the peripheral blood with normal or increased numbers of megakaryocytes in the bone marrow has been explained by a number of theories which are sharply contrasting. As pointed out by Hayem,1 the findings in the disease could be explained equally well on the basis of either a defect of platelet formation or of an excessive disposal or destruction of platelets in the peripheral circulation or in peripheral organs. Several mechanisms have been invoked to explain either possibility. Frank attributed the thrombocytopenia of I.T.P. to decreased formation of platelets by the megakaryocytes,2 probably inhibited in their function by noxious substances produced in the spleen.3 Dameshek and Miller4 later came to somewhat similar conclusions from their studies of the megakaryocyte morphology before and after splenectomy. Kaznelson’s5 suggestion that removal of the spleen might be valuable was based on his concept that the spleen was a thrombocytolytic organ. Doan and his collaborators6 extended these ideas with the concept of selective splenic sequestration of circulating platelets.

Knowledge of the fundamental mechanisms of the disease has recently been advanced through a number of significant observations: (1) elucidation of the mechanism of Sedormid7 and Quinidine8 purpura; (2) the emphasis on the frequent coexistence of I.T.P. and acquired hemolytic anemia9–11; (3) the thrombocytopenic activity of plasma from individuals with I.T.P. when injected into

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† Fellow of the Rockefeller Foundation (1950–1951).
‡ Damon Runyon Clinical Research Fellow.
normal subjects;\textsuperscript{12} (4) the rate of survival of normal platelets transfused into patients with various forms of thrombocytopenic purpura;\textsuperscript{13, 14} (5) studies on the beneficial effect of ACTH and cortisone in the disease\textsuperscript{15–21} and on the congenital thrombocytopenia of the newborn.\textsuperscript{22} It has gradually become evident that I.T.P. is a protean disease, in which the pathogenetic mechanisms may be widely different, thus requiring specialized study from case to case. Particular emphasis has been placed on indications in some cases that circulating substances are present that are capable of attacking platelets directly and, possibly, of inhibiting the production of platelets from the megakaryocytes.\textsuperscript{12, 13}

We have recently studied a patient with "chronic" I.T.P. whose serum contained a platelet agglutinin of high titer. This finding confirms the possibility of detecting platelet agglutinins in the serum of patients with I.T.P., as described by Evans et al.\textsuperscript{11} and, with more exacting technics, from this laboratory.\textsuperscript{23} This case, moreover, is of unusual interest in that the extremely high titer of the platelet agglutinin afforded a unique opportunity for its characterization and purification, as well as for the study of the mechanism of its action, and of the effect of various therapeutic measures on its concentration and activity.

\textbf{Case Report (fig. 1)}

M. K., a 60 year old female textile worker, was first admitted to the New England Center Hospital on November 27, 1951, complaining of the frequent occurrence of purpura, ecchymoses and occasional spontaneous bleeding from the gums for the past three or four years. In October 1950 she had observed fatigue, pallor, weakness and mild jaundice for approximately one month. A similar episode occurred in October 1951, accompanied at this time by puffiness of the eyelids and face, ankle edema and shortness of breath. Severe epistaxis had occurred approximately one week prior to admission. On physical examination there was pallor, questionable icterus of the sclerae and scattered ecchymosis. The spleen was not palpable and appeared of normal size at x-ray examination. The red blood cell count was 3.69 million; hemoglobin 7.8 Gm. per cent (56 per cent); reticulocytes 5.6 per cent; white blood cells 4,400 with 65 per cent polymorphonuclears, 23 per cent lymphocytes, 11 per cent monocytes, and 1 per cent eosinophiles. The platelet count was 51,000 per cu. mm. Most of the platelets were unusually large in size and had very bizarre shapes; some were nongranular. Bone marrow aspiration revealed a normocellular preparation, showing slight normoblastic hyperplasia and lymphocytosis and an increased number of megakaryocytes; of these only 5 per cent appeared to be producing platelets. The number of intermediate megakaryocytes and of promegakaryocytes appeared increased, and some of them appeared to be producing large and bizarre platelets (fig. 2). Total bilirubin was 0.8 mg. per cent; sedimentation rate 5 mm. in one hour. A cephalin-cholesterol flocculation test was 4+; thymol turbidity 3.1 units. No iso-, auto-red cell agglutinins nor hemolysins were detected at 3, 22, 37 C. and the Coombs test was negative. Fecal urobilinogen output was 93 mg. per day. The results of the tests of hemostatic function performed at various intervals of time are summarized in figure 1.

Because of the history of bleeding from the gums and ecchymoses over a period of three or four years, the physical findings and the megakaryocytic type of thrombocytopenia, a diagnosis of chronic I.T.P. was made; it was thought that the mild hypochromic anemia was probably due to chronic blood loss. It was decided that the patient should be followed with periodic platelet counts and the progress of the anemia observed. She was discharged on December 3, 1951 with the recommendation to take ferrous sulfate in a dosage of 0.3 Gm. three times daily. Treatment with iron was continued until January 22, 1952. At this time an increase in the ecchymoses was noted. Cortisone therapy was then begun on January 25, 1952, and continued until March 2, 1952, in a dosage of 150 mg. a day. The spontaneous bleeding manifestations diminished and the capillary fragility became greatly improved,
Fig. 1.—Clinical course of the patient discussed in this article.
but no change in the platelet count was noted. On suspension of therapy, the tourniquet test again became strongly positive and the spontaneous bleeding recurred. Splenectomy was decided upon and performed on March 19, 1952.* A suspension of approximately 800 billion isolated platelets, prepared with a technic to be published24 was given shortly before the beginning of the operation. A moderately enlarged spleen (380 Gm.) was found, with a smooth, glistening capsule. A small thin walled multilocular cyst (1.3 x 1 cm.) containing a small amount of clear fluid emerged from the costal surface of the capsule. The liver appeared slightly enlarged, with a few lobules suggesting a mild cirrhotic process. No biopsy was taken on account of the severe bleeding. Sections of the spleen (formalin fixed tissue) showed multiple anomalous subcapsular cysts, bordered by compressed splenic tissue,

Fig. 2—The bone marrow and megakaryocytes in the patient M. K.

A—Low power bone marrow field (Wright-Giemsa stain; X 80)
B—Promegakaryocyte (from a color Kodachrome; X 570; Leitz Apochromat objective, 1/5 of a second, 0.6 neutral gray filter). Shows complete disappearance of granularity of the cytoplasm.
C—Intermediate megakaryocyte (same). Shows scanty granularity of the cytoplasm with absence of platelet formation.
D—Adult megakaryocyte (same). Shows sparse granulations in the cytoplasm, but without tendency to aggregation of the granules. There is no evidence of platelet formation.
E—Adult megakaryocyte (same). Same appearance of (D).

lined by flattened endothelial-like cells filled with an albuminous coagulum and in part walled off by scar-like sheets of collagen. The splenic pulp itself showed widened sinuses with prominent endothelial lining cells and contained numerous polymorphonuclear leukocytes, together with fairly abundant cells suggesting primitive leukocytes and red blood cells. A few megakaryoblasts were seen. Erythrophagocytosis was not increased and there were little hemosiderosis and lymphoid hyperplasia. Zenker fixed tissue showed considerable myeloid metaplasia. These findings were thought consistent with a diagnosis of I.T.P.†

The postoperative course was uneventful. Although no effect on the platelet count was noticed until the fourth day, and the bleeding time and tourniquet test were still abnormal,
clot retraction became normal almost immediately. On the tenth postoperative day capillary fragility was normal, and the platelet count had risen to 600,000/cu.mm., but the bleeding time remained markedly prolonged. Shortly thereafter, however, the platelet count began to drop and reached the level of 286,000/cu.mm. on April 8, when the patient was discharged. No new hemorrhagic manifestations had been noticed since the day of splenectomy.

The patient's course was then followed at frequent intervals. The platelet count fell progressively to a low value of 160,000/cu.mm. where it apparently became stabilized. The percentage of abnormal forms of platelets (approximately 90 per cent before splenectomy) was unmodified by the operation. The tourniquet test remained negative, clot retraction normal but the bleeding time remained markedly prolonged. The patient felt well and failed to notice any new bleeding manifestations. This status was not modified by the further administration of cortisone (150 mg. a day) or of corticotropin (100 units a day) for a period of 19 and 22 days respectively.

STUDIES ON THE MECHANISM OF THE THROMBOCYTOPENIA IN THE PATIENT

(a) Basic Technics

1. Collection and Administration of Blood, Plasma and Serum

When preservation of the platelets or of the other formed elements of the blood was no consideration, the collection and administration of blood, plasma and serum were carried out by standard procedures under sterile conditions.

2. Platelet Count

This was determined by the indirect method of Dameshek. The results obtained were considered valid only if they were in relative agreement with those of a direct method and when the counts of two separate samples agreed within 5 per cent. To minimize the error due to individual variations, platelet counts were performed by a single observer through the course of this study.

3. Tests for the Study of the Hemostatic Mechanism

The procedures employed are those described in previous communications.

4. Collection and Preparation of Serum for the Study of Antiplatelet Activity

Blood was collected from the donor with the "2-syringe technic" using silicone coated needles and syringes to prevent platelet agglutination. It was then transferred to silicone coated test tubes kept in a constant temperature water bath at 37 C. and allowed to clot. One hour after completion of coagulation the clot was gently detached from the wall of the test tube by means of a paraffin coated glass rod and the tube centrifuged at 2000 r.p.m. for 10 minutes to separate the serum; this was divided in 2 ml. aliquots and stored at -20 C. until used. At the time of use, the serum was thawed at 37 C. for 15 minutes and then inactivated by heating at 56 C. in a constant temperature water bath for 30 minutes. In order to prevent nonspecific agglutination and damage of platelets which might follow if thrombin develops from coagulation factors still present in serum, it was decalcified either by treatment with cation-exchange resin (IRC-50 or Amberlite 1R-100, 1 Gm. to 10 ml. of serum) or by the addition of 1/10 volume of sodium oxalate 0.1 M. and centrifugation at 2000 r.p.m. for 10 minutes. In many cases the serum was also deprothrombinized by treatment with

* Using 1 per cent Sequestrene Na₂ in sodium chloride solution as the diluting fluid.
tricalcium phosphate gel 0.008 M. It was found that the platelet agglutinating activity of the patient's serum was neither affected by storage at −20 C. over a period of at least four months nor by recalcification. Deprothrombinization, on the other hand, decreased the agglutinating activity slightly. Thus, in a typical experiment, the original platelet agglutinating titer of the patient's serum which was 1/4096, remained unmodified after inactivation and decalcification, but was reduced to 1/2048 after treatment with tricalcium phosphate gel.

5. Preparation of Platelet Suspensions for Study of the Platelet Agglutinating Activity of Serum

Blood was collected with the 2-syringe technic in silicone coated syringes through silicone coated needles and transferred to silicone coated test tubes containing 1/10 volume of 1 per cent solution of Sequestrene-Na₂.*

The test tubes were centrifuged for 20 minutes at 500 r.p.m., then left at 4 C. for 2 hours to complete the sedimentation of the red cells. The supernatant plasma was transferred to new test tubes, centrifuged from 2 to 3 times at 500 r.p.m. for 5 minutes to separate as many of the remaining red cells as possible. Three-fourths of the supernatant plasma was then transferred to new test tubes and the plasma centrifuged at 1500 r.p.m. for 20 minutes to precipitate many of the platelets. All operations were carried out in a refrigerated centrifuge at 4 C., using silicone coated glassware. Prior to centrifugation, 1/10 volume of a 2 per cent solution of Tween-80† or, more recently, of Triton WR-1339‡ as suggested by Minor and Burnett§ was added to the plasma. These surface-active agents appear to prevent irreversible clumping of platelets during successive centrifugations. The supernatant plasma was then poured off and the walls of the test tube carefully dried with filter paper. The button of platelets was resuspended in equal volume of saline solution containing 0.2 per cent sodium acetate and 1/10 volume of 2 per cent Triton WR-1339 added. The platelets were then again separated by centrifugation at 1500 r.p.m. for 10 minutes at 4 C. and resuspended in a volume of saline-acetate solution equal to 1/10 of that of the plasma originally collected. Centrifugation at higher speed and further manipulations appeared to cause increasing agglutinability of the platelets by sera. The preparation was then allowed to settle at room temperature for 15 minutes to obtain the separation of any gross clumps present and the supernatant 2/3 transferred to new silicone coated test tubes. The platelet count of the suspension was then obtained by a direct method∥ and the platelet preparation diluted with saline-acetate solution to a count of 300,000 platelets/cu.mm. Only preparations were accepted which failed to show clumps on microscopic observation and contained less than two red and white blood cells per high power microscopic field (fig. 5a). The preparation was kept in the refrigerator at 4 C. and, in these experiments, used within 2 hours.

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* Sequestrene-Na₂ 1 Gm.; NaCl 0.7 Gm.; distilled water to 100 ml. Sequestrene (Disodium ethylenediamine acetate dehydrate) was kindly supplied by the Alrose Chemical Company, Providence, R. I.

† Supplied by Rohm and Haas, Philadelphia, Pa. Eighty ml. of saline solution are heated to 60 C., 2 ml. of Tween-80 or Triton WR-1339 are added and dissolved by rigorous stirring. The volume of the solution is brought to 100 ml. with saline after cooling at room temperature. The solution is sterilized and stored at 4 C.

∥ Supplied by Rohtn and Haas, Philadelphia, Pa. Eighty ml. of saline solution are heated to 60 C., 2 ml. of Tween-80 or Triton WR-1339 are added and dissolved by rigorous stirring. The volume of the solution is brought to 100 ml. with saline after cooling at room temperature. The solution is sterilized and stored at 4 C.
Platelet rich plasma was also used in all these experiments. Blood was collected in silicone coated test tubes containing \( \frac{1}{10} \) volume of 1 per cent Sequestrene-Na\(_2\) solution and centrifuged for 20 minutes at 500 r.p.m. at 4 C. The supernatant plasma was transferred to a new silicone coated tube and again centrifuged at 500 r.p.m. for 10 minutes at 4 C. to separate many of the remaining white and red cells. The platelet count of this preparation was adjusted to 500,000/cu.mm., when necessary, by diluting with platelet poor plasma. This was obtained from the same donor. The silicone coated tube containing the blood originally collected after separation of platelet rich plasma was centrifuged at 4000 r.p.m. for 60 minutes at 4 C, and the platelet poor plasma separated and kept at 4 C. until used. The use of platelets in plasma from a donor of group O was found to give very satisfactory results and avoid the aspecific positive agglutination reactions met with when the serum used is fresh and untreated. It also made the microscopic reading simple and reliable.

Other more specialized technics which were used in the course of this study will be described below.

(b) Studies in Vitro

1. Demonstration of a Potent Human Platelet Agglutinin in the Patient's Serum

(A) Macroscopic agglutination. Platelet rich plasma was obtained as described from compatible donors. Serum from M. K. (our patient) and from other patients (all fasting for the past five hours) was separated from the clot, inactivated, decalcified and deprothrombinized. Two-tenths ml. of our patient's serum was added to 0.8 ml. of compatible platelet rich human plasma in silicone coated test tubes and the mixture kept at room temperature. At the end of three hours, most of the platelets had sedimented to the bottom of the tubes and were there visible as a compact button. The platelet rich plasma mixed with serum of other patients was still opaque at the end of four hours (fig. 3) and no button of platelets...
could be seen at the bottom of the tube. Figure 4 confirms the observation by recording the percentage of transmission of light through the mixtures of normal and patient’s serum and platelet rich plasma at various intervals of time.

(B) Microscopic agglutination. A saline suspension of platelets prepared as described in the previous section was introduced under a cover slip and observed with the phase contrast microscope (fig. 5a). Inactivated, decalcified, deprothrombinized serum of M. K. was then added through one of the sides by capillarity. At the point of contact with serum, the platelets quickly lost their mobility, congregated and formed agglutinates which could not be resuspended by tapping the slide or introducing saline under the coverslip. The appearance of

![Graph showing percentage transmission of platelet suspensions in plasma at various intervals of time after mixing with normal serum and serum of our patient.](image)

**Fig. 4—**Per cent transparency of platelet suspensions in plasma at various intervals of time after mixing with normal serum and serum of our patient. The prompt agglutination and sedimentation of platelets incubated with our patient’s serum is indicated by the quick increase of transparency of the suspension.

the field under phase contrast microscopy changed completely in the space of a few seconds from that of figure 5a to that of figure 5b. It was also observed that platelets tended to migrate to the area where the patient’s serum had been introduced and promptly agglutinate there. This test was repeated several times using sera of normal individuals and patients with I.T.P. In 6 out of 45 cases of I.T.P. examined, all of the chronic variety and in whom a platelet agglutinin could be demonstrated by other tests as well, a result similar to that observed in M. K. was obtained, although the degree of clumping was much less marked.

Suspensions of platelets from dog, rabbit, sheep and hamster were also prepared and tried against M. K.’s serum. Results were equivocal, and there was no prompt or massive clumping of platelets as in the case of human platelets.

(C) Determination of the agglutinating titer of the serum. One-tenth ml. of platelet suspension prepared from a compatible donor and 0.1 ml. of M. K.’s
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treated serum at various dilutions with saline were transferred by means of silicone coated pipets to 8 x 50 mm. serology tubes coated with silicone. At the same time, plasma rich in platelets, obtained as described, was also used. Two control test tubes were also prepared: the first one, containing 0.1 ml. of buffered saline and 0.1 ml. of platelet suspension, and the second, containing 0.1 ml. of platelet suspension and 0.1 ml. of normal human serum treated as described. The test tubes were incubated at room temperature and the results read after 90

FIG. 5 A—Preparation of platelets in saline suspension, obtained with the method described in this paper. (Magnification 400 X, dark contrast phase objective, 1/2 of a second.)

B—Same field of figure 5 A, after the introduction of M. K.'s serum under the slide. (Magnification 400 X, dark contrast phase objective, 1/2 of a second.)

minutes. The platelets were resuspended by gently tapping the bottom of the tube. Presence of clumping was observed macroscopically under a strong light over a concave lens giving a ×3 enlargement, and microscopically under high dry lens. Small drops of silicone were at times observed in the field, but they did not interfere with the reading. The titer given was the dilution of the serum in the last tube showing microscopic agglutination.

All experiments were run in duplicate and, in order to minimize the error of the technic, the results were always read by the same two observers and accepted only if identical. This method has been thus far applied to 155 normal sera
kept at $-20\,\text{C.}$ for at least twenty days and inactivated, decalcified and depro-
thrombinized prior to use, and uniformly negative results have been obtained.

(D) Demonstration of a "coating" agglutinin on M. K.'s platelets. Following
splenectomy, the platelet count rose temporarily to a level of over
600,000/cu.mm. At this time blood was collected from the patient, platelets
separated by the technic previously described, and washed three times with
buffered saline solution. One-tenth volume of 2 per cent Triton WR-1339 was
added each time to limit clumping of platelets on resuspension. The final button
of platelets was then resuspended by agitation in a small volume of the Coombs
antiglobulin (human) serum at various dilutions. Two sera were used: one was
a commercial preparation and the other prepared in this laboratory by the
injection of human serum. Control tests were performed using platelets similarly
obtained from a healthy, nonthrombocytopenic individual, and from another
patient with chronic I.T.P. After one hour of incubation at $37\,\text{C.}$, M. K.'s plate-
lets were strongly agglutinated by the serum at a final dilution of $\frac{1}{32}$. No
agglutination was noted in any of the control tests.

It appears, therefore, that the isolated platelets of this patient were coated
by a substance causing their agglutination when incubated against antihuman
serum prepared according to the technic of Coombs. This is, to our knowledge,
the first demonstration of the presence of a platelet agglutinating agent adsorbed
on the surface of the blood platelets.

(E) Role of complement in the agglutinating activity of M. K.'s serum. Suspensions
of platelets in saline (1,000,000/cu.mm.) were prepared from a healthy
O,CDe donor. At the same time blood collected by silicone technic in $\frac{1}{40}$ volume
of 1 per cent Sequestrene was centrifuged at 4000 r.p.m. for 1 hour at 4 C. to
make the plasma as platelet poor as possible. Mixtures were prepared in silicone
coated test tubes of 0.1 ml. of platelet suspension, 0.1 of inactivated M. K.'s
serum and 0.2 ml. of platelet poor plasma as source of complement. Platelet
counts were taken immediately and after 90 minutes and 3 hours of incubation
at room temperature. Control tests were performed using inactivated serum from
normal individuals. A reduction of 60 to 70 per cent from the original platelet
count was observed in the tubes containing M. K.'s serum after 90 minutes
incubation. It is unlikely, however, that such a result indicated actual lysis of
platelets, since gross agglutination was present in those test tubes and similar
results were obtained when serum and platelets were incubated alone without
plasma.

Confirmatory evidence was found in complement fixation tests which were
performed independently by two separate investigators and which failed to give
any consistent indication of utilization of complement during the reaction of
agglutination.

2. The Inhibitory Effect of the Patient's Serum on the Activity of Normal Platelets

(A) Effect of M. K.'s serum on the degree of clot retraction of normal native
plasma. Blood was collected from individuals of blood groups O, A, B, and AB
in silicone coated chilled test tubes, by the 2-syringe technic. Blood was then
spun at 1000 r.p.m. for 10 minutes in a refrigerated centrifuge at 4 C. By means
of chilled silicone coated pipets the supernatant native plasma containing most
of the platelets was quickly transferred to silicone coated test tubes kept at 4 C. and used as soon as possible. The platelet count was adjusted to 500,000/cu.mm. as calculated by a direct method by diluting the platelet rich plasma with previously obtained plasma from the same donor made platelet poor by centrifugation at 4000 r.p.m. for 1 hour in the refrigerated centrifuge at 4 C. Sera used included two from normal individuals, two from patients with I.T.P. not exhibiting platelet agglutinating activity and the serum of the patient under study. The serum was separated from blood, allowed to clot at 37 C., the clot being incubated for 1 hour at 37 C. The serum was then kept at room temperature for 5 hours before use. As soon as possible after preparation of the platelet rich human native plasma, 0.3 ml. of each serum was transferred to glass test tubes kept in a water bath at 37 C. Seven-tenths of native platelet rich human plasma was quickly added to each tube. The tubes were promptly and repeatedly tilted to assure good mixing of plasma and serum, and observed for clotting. Clotting, as indicated by complete opacity of the plasma, occurred promptly in 1½ to 2 minutes in all tubes but the one containing M. K.'s serum, where it took between 3 and 5 minutes. Clot retraction was observed at the end of 1 hour. As seen in figure 6 retraction of the clot was incomplete and a large mass of fibrin could be seen settling at the bottom in the central tube which contained native plasma mixed with M. K.'s serum. In the two tubes to the left and the two to the right containing respectively 2 sera from patients with I.T.P. not exhibiting platelet agglutination activity and 2 normal individuals, retraction of the clot was complete.

(B) Effect of the patient's serum on the prothrombin utilization during the clotting of normal human native plasma. Blood was collected from a compatible donor with the 2-syringe technic and using glassware and needles coated with silicone. It was then centrifuged in the cold (4 C.) at 500 r.p.m. for 15 minutes and the supernatant plasma, containing most of the platelets, transferred to a chilled silicone coated test tube. Three-tenths ml. of decalcified and depo-thrombinized patient's serum were added to 0.7 ml. of platelet rich native plasma and the mixture allowed to clot in a water bath at 37 C. The residual prothrombin activity of serum was determined 1 hour after completion of clotting by the method of one of us. Controls were run with the use of sera from normal individuals and sera from patients with I.T.P. which did not exhibit platelet agglutini-

![Image](image-url)
nating activity. As shown in table 1, the residual prothrombin activity of serum was always lower in the mixture containing M. K.’s serum than in the mixture containing sera from normal individuals or patients with I.T.P. The conclusion suggests itself that while the patient’s serum, by agglutinating the platelets, decreased their ability to induce normal clot retraction, on the other hand it apparently caused more complete release from the platelets of factors influencing the activation of thromboplastin and, consequently, the amount of prothrombin utilized during clotting. A number of factors have been found in platelets which are able to accelerate the activation of thromboplastin and the conversion of prothrombin to thrombin. (For review see the article by Stefanini.33)

3. Investigation of Some of the Properties of the Platelet Agglutinin

M. K.’s serum was inactivated, decalcified and deprothrombinized. It was finally centrifuged at 3000 r.p.m. for 15 minutes at 4 C. to separate all particulate matter, only the 3/4 supernatant being used. The serum was then submitted to various procedures to establish some of the properties of the platelet agglutinin as shown by changes in its titer against normal human platelets. The results obtained in the course of this investigation are presented in table 2.

(A) Procedures failing to influence the titer of platelet agglutinating activity of M. K.’s serum were: (1) storage of the serum at -20 C. for a period of two months; (2) heating of the serum at 56 C. for 30 minutes (“inactivation”); (3) passage through Seitz filter. This procedure, due in fact to the concentration of the specimen, yielded serum with an agglutinating titer higher than that of the original serum; (4) dialysis against buffered saline (pH 7.0) at 4 C. for 24 hours. The remaining serum was then lyophilized and reconstituted to the original volume with distilled water; (5) decalcification of the serum by the addition of 3/10 volume sodium oxalate 0.1 M or by passage through a column of a cation exchange resin (1 RC-50 or Amberlite IR-100: 1 Gm. per 10 ml. of serum).

(B) Procedures minimally affecting the platelet agglutinating titer of the serum were: (6) adjustment of the pH of the serum to pH 9.0 by means of addition of 5N NaOH solution, or to 5.0 by means of the addition of 3N HCl solution;

<table>
<thead>
<tr>
<th>Serum Added</th>
<th>Platelet Rich Plasma</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Clot Retraction %</td>
</tr>
<tr>
<td>Patient’s serum (M. K.)</td>
<td>12</td>
</tr>
<tr>
<td>Serum of patient with I.T.P.†</td>
<td>37</td>
</tr>
<tr>
<td>Serum of patient with I.T.P.†</td>
<td>42</td>
</tr>
<tr>
<td>Serum of patient with aplastic anemia</td>
<td>38</td>
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<tr>
<td>Serum from healthy individual</td>
<td>44.5</td>
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<tr>
<td>Serum from healthy individual</td>
<td>39</td>
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</tbody>
</table>

* Three-tenths ml. of serum were added to 0.7 ml. of human platelet rich native plasma. Serum had been inactivated, decalcified and deprothrombinized prior to use.
† Nonexhibiting platelet agglutinating activity in vitro.
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(7) absorption with equal volume of compatible packed human red cells at 37 C. for 4 hours and separation of the serum by centrifugation at 3000 r.p.m. for 15 minutes; (8) absorption with equal volume of packed beef erythrocyte antigen at room temperature for 2 hours, and separation of the serum by centrifugation at 3000 r.p.m. for 15 minutes at room temperature; (9) deprothrombinization of the serum by the technic previously described.

(C) Procedures greatly affecting the platelet agglutinating titer of the patient's serum were: (10) Storage of the serum at room temperature for two months in tubes sealed with paraffin; (11) heating of the serum at 60 C. for 15 minutes in a constant temperature water bath; (12) adjustment of the pH to 3.0 by means

Table 2—Effect of Various Treatments of the Patient's Serum on the Activity of the Platelet Agglutinin

<table>
<thead>
<tr>
<th>Treatment Description</th>
<th>Titer against Human Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum, stored for 2 months at -20 C.</td>
<td>1/2048</td>
</tr>
<tr>
<td>Serum, stored for 2 months at room temperature</td>
<td>1/256</td>
</tr>
<tr>
<td>Serum, heated at 56 C. for 30 minutes</td>
<td>1/2048</td>
</tr>
<tr>
<td>Serum, heated at 60 C. for 15 minutes</td>
<td>1/512</td>
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<tr>
<td>Serum, heated at 65 C. for 15 minutes</td>
<td>0</td>
</tr>
<tr>
<td>Serum, pH 9.0</td>
<td>1/1024</td>
</tr>
<tr>
<td>Serum, pH 7.0</td>
<td>1/2048</td>
</tr>
<tr>
<td>Serum, pH 6.0</td>
<td>1/1024</td>
</tr>
<tr>
<td>Serum, pH 5.0</td>
<td>1/256</td>
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<tr>
<td>Serum, pH 3.0</td>
<td>1/1024</td>
</tr>
<tr>
<td>Serum, dialyzed for 24 hours at 4 C. against buffered saline, lyophilized and reconstituted to original volume</td>
<td>1/2048</td>
</tr>
<tr>
<td>Serum, absorbed with human red cells</td>
<td>1/1024</td>
</tr>
<tr>
<td>Serum, absorbed with guinea pig kidney antigen</td>
<td>1/512</td>
</tr>
<tr>
<td>Serum, absorbed with beef erythrocyte antigen</td>
<td>1/1024</td>
</tr>
<tr>
<td>Serum, decalcified by addition of 1/10 volume sodium oxalate 0.1M.</td>
<td>1/2048</td>
</tr>
<tr>
<td>Serum, decalcified by passage through cation-exchange resin (IRC-50).</td>
<td>1/2048</td>
</tr>
<tr>
<td>Serum, decalcified and deprothrombinized by absorption with Ca3(P04)2</td>
<td>1/1024</td>
</tr>
</tbody>
</table>

* Sample was concentrated by the procedure.

of the addition of N-3 HCl solution; (13) absorption with equal volume of packed kidney guinea pig antigen at room temperature for 2 hours and separation of the serum by centrifugation at 3000 r.p.m. for 15 minutes; (14) absorption with packed human platelets (see below).

(D) Procedure completely destroying the platelet agglutinating activity of the patient's serum was that of heating the serum at 65 C. for 15 minutes.

4. Absorption of the Platelet Agglutinin by Normal Human Platelets

For these experiments, the patient's serum was decalcified and deprothrombinized. One ml. of serum was mixed with a suspension of approximately 5 billion packed human platelets. The packed platelets were resuspended in the serum, the mixture being incubated at room temperature for 1 hour and then at 4 C. overnight. Merthiolate 1/10 volume of 1:1000 solution was added to the mixture.
to prevent bacterial contamination. The platelets, which were heavily clumped, were then separated by centrifugation at 3000 r.p.m. for 15 minutes and the serum collected and tested for residual platelet agglutinating activity. The entire procedure was repeated six times, the titer of the platelet agglutinin in the serum of the patient decreasing progressively as indicated in table 3. Breakage of the tubes during centrifugation during the seventh absorption experiment prevented further absorption of the platelet agglutinin from the serum. It is likely, however, that since the titer of the agglutinin was rapidly decreasing as repeated absorptions were conducted, further processing could have brought about its complete absorption.

Attempts were also made to elute the agglutinin absorbed on the clumped platelets. The following approach proved successful. The platelets used in the first absorption experiments were washed twice with a saline solution of pH 7.4 at a temperature of 4 °C., to remove most of the nonabsorbed material. The clumped platelets were then resuspended in a volume of saline solution of pH 3.2 equal to half the volume of the serum originally used for absorption experi-

<table>
<thead>
<tr>
<th>No. of Absorptions</th>
<th>Titer of Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>1/4096</td>
</tr>
<tr>
<td>1</td>
<td>1/2048</td>
</tr>
<tr>
<td>2</td>
<td>1/1024</td>
</tr>
<tr>
<td>3</td>
<td>1/512</td>
</tr>
<tr>
<td>4</td>
<td>1/512</td>
</tr>
<tr>
<td>5</td>
<td>1/256</td>
</tr>
<tr>
<td>6</td>
<td>1/64</td>
</tr>
<tr>
<td>7</td>
<td>1/8</td>
</tr>
</tbody>
</table>

ments and the mixture incubated in a constant temperature water bath at 37 °C. The solution was then adjusted to pH 7.0 by the addition of 5N NaOH solution, the platelets separated by centrifugation at 3000 r.p.m. for 2 hours at 4 °C., and the supernatant fluid collected. Saline solution at pH 7 was then added to reconstitute the volume of serum originally employed. The final eluate agglutinated human platelets at a dilution of 1:512. Thus, it was possible to elute the agglutinin from the clumped platelets with a relatively simple technic, although with marked loss of activity. The absorption of the agglutinin on the clumped platelets was further demonstrated by the following experiment. Two samples of a platelet suspension were packed by centrifugation at 3000 r.p.m. for 1 hour at 4 °C. One ml. of M. K.'s serum per 5 billion platelets was added to the platelet button. The platelets were resuspended by gentle agitation and the mixture incubated at room temperature for 1 hour and then at 4 °C. overnight. The platelets of both aliquots were then separated by centrifugation at 3000 r.p.m. for 1 hour at 4 °C., washed three times with saline at 4 °C., the platelets being packed down by centrifugation at 3000 r.p.m. for 1 hour at 4 °C. each time. The nitrogen content was finally determined by the micro-Kjeldahl technic in both aliquots and in a sample containing a known number of untreated platelets. It was calculated
that the individual platelet had absorbed $0.32 \times 10^{-3}$ N after incubation with normal serum and $1.42 \times 10^{-3}$ N with M. K.'s serum. Although this experiment is admittedly open to criticism, the difference in N content between the two samples may represent the abnormal agglutinin present on the platelets.

### Table 4—Nitrogen Content and Platelet Agglutinin Activity of Various Fractions of M. K.'s Serum, as Obtained by Salt Removal Technic

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Nitrogen (mg./100 ml.)</th>
<th>Agglutinating Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble (supernatant)</td>
<td>710</td>
<td>0</td>
</tr>
<tr>
<td>Salt soluble, NaCl ionic strength</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.001 M</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.01 M</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>0.025 M</td>
<td>70.5</td>
<td>0</td>
</tr>
<tr>
<td>0.05 M</td>
<td>250.5</td>
<td>1/16</td>
</tr>
<tr>
<td>0.075 M</td>
<td>178.0</td>
<td>1/16</td>
</tr>
<tr>
<td>0.1 M</td>
<td>125.0</td>
<td>1/64</td>
</tr>
<tr>
<td>0.115 M</td>
<td>60</td>
<td>1/128</td>
</tr>
<tr>
<td>0.15 M</td>
<td>75</td>
<td>1/256</td>
</tr>
<tr>
<td>0.2 M</td>
<td>45</td>
<td>1/512</td>
</tr>
</tbody>
</table>

Fig. 7—Electrophoretic patterns of the patient’s serum. A. Ascending boundary; B. descending boundary. (Veronal citrate buffer, pH 8.6; ionic strength $\mu = 0.1$; electric field 8.8 volts/cm; time 4200 seconds.) Serum centrifuged at 40,000 r.p.m. at 4 C. for 1 hour prior to dialysis.

5. **Separation of Platelet Agglutinin by Salt Removal Technic**

The technic, a modification of the procedure of Reed and Jones, has been previously described. By this technic (table 4) the platelet agglutinin was found to be present in the water insoluble, salt soluble globulin fraction of plasma. High concentrations of sodium chloride were required for the best recovery of the agglutinin, the highest agglutinin titer being found in the globulin fraction soluble.
in 0.2 M sodium chloride. The amount of protein in each fraction was too low to allow electrophoretic analysis of each fraction.

6. Electrophoretic Pattern of M. K.'s Serum and Plasma

Electrophoretic ascending and descending boundaries were obtained of M. K.'s plasma or serum, using veronal-citrate buffer at pH 8.6, ionic strength $\mu = 0.1$, electric field 8.8 volts/cm., time 4200 seconds. Since sera often appeared turbid prior to electrophoresis, they were centrifuged at 40,000 r.p.m. for 1 hour. This procedure failed to reduce the platelet agglutinating titer. The electrophoretic patterns obtained remained almost identical both before and after splenectomy and through the nine months period of observation (fig. 7a and b). An abnormally high concentration of protein was observed in the $\beta_2$ area (table 5) possibly representing the abnormal agglutinin. By an electrophoretic separation process (to be reported elsewhere) it was possible to isolate this protein as an electrophoretic homogenous component with high platelet agglutinating activity.

(c) Studies in Vivo

1. The Survival Time of Platelets Injected in the Circulation of M. K.

Platelets were prepared by two different methods. In one, blood was collected from a compatible polycythemic donor in $\frac{1}{30}$ volume of 1 per cent Sequestrene solution in silicone coated containers* and through plastic tubing and silicone coated needles. Blood was centrifuged at 1000 r.p.m. for 20 minutes to separate most of the red cells and the plasma transferred to a plastic container. The platelet rich plasma was then injected into the patient at the approximate speed of 1 cu. mm. per minute, for an approximate total of 625 billion platelets, calculated to produce a theoretical rise in the platelet count of 120,000/cu. mm. The

Table 5—Electrophoretic Characteristics of the Patient's Serum

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rising Boundary (cm²/volt·sec⁻¹)</th>
<th>Descending Boundary (cm²/volt·sec⁻¹)</th>
<th>Normal (cm²/volt·sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>$5.9 \times 10^{-5}$</td>
<td>$5.9 \times 10^{-5}$</td>
<td>$5.92 \pm 0.21 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\alpha_1$-globulin</td>
<td>$5.25 \times 10^{-5}$</td>
<td>$5.15 \times 10^{-5}$</td>
<td>$4.85 \pm 0.23 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\alpha_2$-globulin</td>
<td>$3.9 \times 10^{-5}$</td>
<td>$3.8 \times 10^{-5}$</td>
<td>$3.87 \pm 0.22 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\beta_1$-globulin</td>
<td>$2.9 \times 10^{-5}$</td>
<td>$2.8 \times 10^{-5}$</td>
<td>$2.88 \pm 0.15 \times 10^{-5}$</td>
</tr>
<tr>
<td>$\beta_2$-globulin</td>
<td>$1.7 \times 10^{-5}$</td>
<td>$1.2 \times 10^{-5}$</td>
<td></td>
</tr>
<tr>
<td>$\gamma$-globulin</td>
<td>$0.9 \times 10^{-5}$</td>
<td>$0.8 \times 10^{-5}$</td>
<td>$1.15 \pm 0.20 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

(B) Concentration (per cent)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>58.78</td>
</tr>
<tr>
<td>$\alpha_1$-globulin</td>
<td>3.77</td>
</tr>
<tr>
<td>$\alpha_2$-globulin</td>
<td>6.97</td>
</tr>
<tr>
<td>$\beta_1$-globulin</td>
<td>13.28</td>
</tr>
<tr>
<td>$\beta_2$-globulin</td>
<td>9.33</td>
</tr>
<tr>
<td>$\gamma$-globulin</td>
<td>7.87</td>
</tr>
</tbody>
</table>

* Especially prepared by the Baxter Company, Morton Grove, Ill.
experiment was performed one month before and one month after splenectomy and the results obtained were closely similar (fig. 8). Although the platelet count rose during the transfusion, the peak increment was far short of the expected rise; and there was even a fall beginning while the platelets were still being administered. The platelet level was, as a matter of fact, lower than the original value at the end of the experiment and some time after the completion of the transfusion, returning to the pre-experiment level approximately 2 hours later. It is possible that this secondary thrombocytopenic effect was comparable to the temporary drop in the platelet count observed when normal plasma is injected into many healthy recipients. While, however, the platelets disappeared very promptly from the circulation in the presplenectomy experiment, the capillary fragility and utilization of prothrombin during clotting temporarily
become normal but returned to original values within 4 hours. The bleeding
time was unaffected.

The tourniquet test became negative and the prothrombin utilization during
clotting improved following splenectomy. Platelet transfusions at this point
increased the prothrombin utilization for approximately 4 hours, but again failed
to modify the bleeding time, which had remained abnormally prolonged. Very
similar results were obtained by administering blood from a polycythemic donor
collected in an entirely plastic system.

An identical set of experiments was conducted prior to and 40 days after
splenectomy, but administering isolated platelets resuspended in saline. Using
silicone coated needles, bottles, and plastic tubing for all procedures, blood was
collected by gravity from 4 O-Rh+ donors (compatible with M. K.) in \(\frac{1}{10}\)
volume of 1 per cent Sequestrene-Na2 solution and the plasma separated by cen-
trifugation in the cold at 1000 r.p.m. for 30 minutes. The four aliquots of plasma
obtained were then pooled and transferred to two new silicone coated bottles.
These were centrifuged again in the cold at 1000 r.p.m. for 15 minutes, to sepa-
rate most of the remaining red and white blood cells. At this point \(\frac{1}{10}\) volume
of 2 per cent solution of Tween-80 or Triton 1339-WR in saline was added to
the plasma, and the platelets packed by centrifugation in the cold at 3000 r.p.m.
for 30 minutes. The supernatant plasma was siphoned off, saline solution equal
to \(\frac{1}{4}\) of the volume of the plasma originally used was then added to the packed
platelets, these resuspended in saline. All preparations were finally pooled in
one silicone coated container. A total number of 625 billion platelets were again
administered to the patient in two separate experiments before and after sple-
enectomy, using plastic tubes and silicone coated needles. While the effects on
the capillary fragility, prothrombin utilization during clotting and bleeding time
were essentially similar to those obtained with platelets suspended in plasma, we
failed to observe even a temporary rise in the platelet count during or after the
transfusion. This result is in agreement with other unpublished observations from
this laboratory indicating that platelets prepared by any method involving
repeated centrifugation or resuspension in saline, although capable of improving
the bleeding tendency are usually unable to increase, even temporarily, the
platelet level in thrombocytopenia.

The results with platelet-rich polycythemic blood and plasma, however, sug-
gest that the survival of platelets is greatly decreased in the presence of a circulat-
ing platelet agglutinin. The number and appearance of abnormal platelets was
carefully studied before and after the transfusion, and remained essentially the
same. This finding suggests that either the injected platelets failed to remain in
the circulation or became modified in appearance through the activity of the
platelet agglutinin. This problem will be discussed further in the course of this
paper.

2. The Effect of the Administration of the Patient's Plasma into Healthy Recipients

The administration of plasma from some patients with I.T.P. to healthy
recipients is followed by significant thrombocytopenia.\(^2\) This effect varies greatly
from donor to donor and, in our series of 35 patients, the plasma of only 2 cases
of I.T.P. (including M. K.) appeared capable of causing a consistently severe
thrombocytopenia and purpura in healthy recipients. In 12 other cases a reduction of approximating 50 per cent in platelet count took place. This could be distinguished only in duration from the transient thrombocytopenia which follows the administration of plasma to many normal recipients. In the course of various observations, a group of 15 compatible recipients over a period of six months received M. K.'s plasma. If not otherwise stated, each recipient received plasma to an amount of 3 ml. of M. K.'s plasma/Kg. of body weight. This dose consistently caused severe thrombocytopenia and clinical purpura when given at the speed of 10 ml. per minute.

![Graph](image)

**Fig. 9**—The effect of the administration of M. K.'s plasma to a normal individual on the platelet count, platelet agglutinin titer of the serum, number of inactive megakaryocytes and abnormal platelets.

(A) *Effect on the platelet count* (Figure 9). Even while the plasma was being administered, the platelet count of the recipient fell to a very low level (10,000 to 20,000/cu.mm.). The platelet count remained at these low levels for 1 to 3 days and rose slowly to return to normal values in approximately 7 to 10 days. During the first day of observation, purpura (petechiae over arms, trunk and legs) was regularly noted.

The platelets of both patient M. K. and recipients were observed in fixed preparations stained with Giemsa for size, appearance and granularity. As shown in figure 10, the platelets of M. K. were definitely abnormal, varying greatly in size, appearance, granularity, etc. Some appeared long and slim, some
curved, some round; all were generally larger than normal and few even larger than a red blood cell. The hyalomere appeared large, irregular and with many pseudopods. The chromomeres were large, few with heavy granulations, many with reduced granularity or almost agranular.

When healthy recipients received M. K.'s plasma, the platelet count began to rise after 2 or 3 days. At this time the appearance of platelets was very similar to that of M. K.'s platelets. Although the platelet count returned to normal

value in approximately 7 days, the platelets still appeared abnormal 12 to 14 days after the administration of M. K.'s plasma (fig. 9).

(B) Survival time of the platelet agglutinin within the recipient's circulation. No agglutinating activity against platelets could be demonstrated in one recipient's serum prior to the administration of M. K.'s plasma. Following the transfusion, the recipient's serum exhibited a platelet agglutinin titer of 1/256. This titer dropped to 1/32 on the fourth day, to 1/16 on the ninth day, and a doubtful result was obtained on the thirteenth day (fig. 9). No direct correlation was
established between agglutinin titer of the serum and platelet level of the recipients, since the latter had returned to normal values when a platelet agglutinin titer of 1/16 was still detectable in the serum.

Plasma was collected from 2 recipients at the height of the induced thrombocytopenia and injected 2 ml./Kg. weight into other healthy individuals. The thrombocytopenic response observed was not greater than that often observed following the administration of normal plasma and no platelet agglutinin activity could be detected in the serum of the second recipient.

(C) Coating of the platelets of the recipient by Coombs reacting substance. A nonthrombocytopenic healthy recipient received 1.1 ml./Kg. weight of M. K.'s plasma. Blood was collected from the recipient at the peak of the induced thrombocytopenia (approximately 24 hours after the administration of M. K.'s plasma, Case No. 1 in fig. 11). A suspension of platelets in saline was prepared.
by the method previously described and tested against Coombs antiserum as
described in an earlier paragraph. Although no platelet agglutinin could be
detected in the recipient's serum, the recipient's platelets were agglutinated on
incubation with Coombs serum (a commercial preparation and one obtained in
our laboratory) in a final dilution of 1:8. Platelet suspensions prepared from
healthy individuals were not agglutinated.

(D) Morphologic changes in the megakaryocytes of the recipient following ad-

ministration of M. K.'s plasma. To evaluate the morphologic changes produced
in the megakaryocytes by the administration of M. K.'s plasma, 3 healthy

recipients were given an amount of this plasma capable of inducing a moderate
thrombocytopenia of one to three days duration (2.0 ml./Kg. weight). Platelet
counts, bleeding time, tourniquet test, utilization of prothrombin during cloting
were determined at various intervals. Simultaneously, bone marrow aspirations
were performed, and smears observed directly for megakaryocyte morphology
either with phase contrast microscopy or after staining with May-Grünwald-
Giemsa. Prior to the administration of plasma (fig. 12a) the megakaryocytes
were mostly of the mature variety, many of them showing a well lobulated
nucleus, granular cytoplasm with granules agglutinated at the periphery and
surrounded by platelets. Thirty minutes after the administration of the patient's
plasma, the characteristics of nucleus and cytoplasm of the megakaryocytes

Fig. 12—Morphologic alterations of the megakaryocytes in a healthy individual following
the administration of M. K.'s plasma.

A—Low power field of the recipient's bone marrow 2 hours after the administration of
M. K.'s plasma ($\times$ 80).

B—Individual megakaryocytes (from color Kodachromes, $\times$ 970, Leitz Apochromat ob-
jective, 0.6 neutral green filter, 1/5 of a second). (Explanation in the text.) 1. (Lower left)
Before the administration of M. K.'s plasma. 2. (Upper right) Twenty minutes later. 3.
(Center) Two hours later. 4. (Lower right) Twenty-four hours later.
were essentially unmodified, but the granules appeared uniformly distributed in the cytoplasm and not, as before, agglutinated at the periphery of the giant cells, most of which were no longer surrounded by platelets (fig. 12b). Two hours later, an increased number of earlier forms (intermediate megakaryocytes and promegakaryocytes) had become evident. Of the mature megakaryocytes, many presented partial or complete loss of granularity and large vacuoles in their cytoplasm and overlying the nucleus (fig. 12c). Twenty-four hours later the proportion of mature to immature megakaryocytes had returned to almost normal values, the cytoplasm appeared granular again and, although only very few or no platelets were visible at the periphery of the cell, granules were beginning to agglutinate at the periphery of the cytoplasm (fig. 12d). The same alterations of the megakaryocytes were observed when the patient's plasma was administered in comparable volume to 2 splenectomized patients. Direct studies on unfixed bone marrow aspirates by phase contrast microscopy (to be published in a separate report) confirmed these observations. These results and those on the effect of M. K.'s plasma on the number of platelets circulating in the recipient, seem to indicate that the thrombocytopenia induced in the recipient may be due to a double mechanism: (1) a direct effect on the platelets in the peripheral circulation, and possibly, even on those on the surface or emerging from the megakaryocytes; (2) damage to the megakaryocyte itself as demonstrated by the lack of granularity or condensation of granules and protoplasmatic vacuolization, with consequent inhibition of platelet production. These two mechanisms and their possible pathogenesis will be given further attention in the discussion.

3. The Influence of Various Procedures on the Agglutinin Titer Demonstrable in Vitro and the Thrombocytopenic Effect in Vivo of M. K.'s Plasma

A. Effect of repeated venesections on the agglutinin titer and thrombocytopenic effect. Five hundred ml. of blood were collected from the patient, M. K., in ACD solution on December 2, 10, 27, 1951, and January 4, 15 and 22, 1952. Immediately following each venesection, 500 ml. of compatible blood were administered as replacement. Samples of serum were simultaneously procured, and after treatment as described in a previous paragraph, the titer of the platelet agglutinin was determined in each. The collected blood was centrifuged at 2000 r.p.m. for 60 minutes at room temperature to separate the plasma, which was then transferred to a vacuum container. This plasma was injected within five days of collection into normal recipients, in a volume of 2.8 ml./Kg. of body weight (fig. 13). As a result of the repeated venesections, the platelet agglutinin titer of the serum decreased from 1/2048 to 1/256 but, notwithstanding this, the platelet count of the patient slowly fell from 150,000 to 78,000/eu.mm. (fig. 1). When the plasma collected from the patient in successive venesections was injected into a series of healthy individuals, it was noted that the immediate drop in the platelet count which followed the administration of plasma was not appreciably affected by the reduction in titer of the platelet agglutinin produced by the repeated bleeding. A direct relation, however, was found between agglutinin titer and duration of the thrombocytopenic effect. The administration of plasma collected on December 2, 1951 (agglutinin titer 1/2048) was followed by thrombocytopenia of five days duration; that of plasma collected on
January 22, 1952 (agglutinin titer 1/256) by thrombocytopenia of approximately half a day.

B. Effect of the administration to the patient of ACTH and cortisone. The patient received cortisone prior to and following splenectomy. The administration of cortisone apparently reduced the platelet agglutinin titer from 1 2048 to 1/512, but, with the exception of a fleeting elevation, failed to modify the platelet count appreciably. Plasma collected at the time of optimal reduction of the agglutinating titer in vitro during cortisone administration produced a slightly less prolonged (three days) thrombocytopenic effect. In view of the variability in the thrombocytopenic response of different recipients given M. K.'s plasma, it is not possible to state categorically that the administration of cortisone had any definite effect in reducing the thrombocytopenic activity in vivo of the patient's plasma.

Following splenectomy, the platelet count of the patient rose temporarily and, ten days after the operation, was 618,000/cu.mm. More or less simultaneously with this increase, the agglutinin titer of the serum fell for a few days. M. K.'s plasma collected at the moment of the highest platelet count appeared unmodified in its ability to produce thrombocytopenia when injected into normal recipients. Two months after splenectomy, the patient was given cortisone in a dosage of 150 mg. daily for nineteen days and corticotropin in a dosage of 100 units daily for twenty-one days. On both occasions, the medication caused a questionable reduction of minor degree in the platelet agglutinating activity of
the serum in vitro, but failed to appreciably modify the thrombocytopenic activity of the patient's plasma in vivo. Repeated small venesections failed, at this time, to affect greatly the platelet agglutinin titer in vitro and the thrombocytopenic activity in vivo of the patient’s plasma.

![Graph showing thrombocytopenic effect of patient's plasma](image)

**Fig. 14**—Thrombocytopenic effect of the administration of the patient’s plasma to normals, normals treated with cortisone and splenectomized individuals.

4. **Attempts to Modify the Thrombocytopenic Response of Normal Recipients to the Administration of M. K.’s Plasma**

   A. **Effect on the administration of corticotropin and cortisone to the recipient on the thrombocytopenic effect of the patient’s plasma.** One healthy individual received 60 mg. of corticotropin intramuscularly and, at the same time, 1.2 ml./Kg. weight of M. K.’s plasma was injected intravenously. Another healthy individual received 150 mg. of cortisone intramuscularly and the same amount of M. K.’s plasma. No evidence was obtained that this maneuver caused any appreciable change in the thrombocytopenic response of normal recipients to M. K.’s plasma.

   B. **Effect of heparin.** The administration of 75 mg. of heparin sodium intra-
venously to a healthy recipient failed to modify appreciably the thrombocytopenic effect of M. K.’s plasma.

C. Thrombocytopenic response of splenectomized individuals. One patient with hereditary spherocytosis received 2.0 ml./Kg. weight of M. K.’s plasma five days prior to and again ten days following splenectomy. The results are indicated in figure 14, which also presents the thrombocytopenic response in a normal recipient and in a patient splenectomized five years previously for Mediterranean anemia. It is evident that removal of the spleen, whether recent or of many years standing, shortened to the same extent the duration of the thrombocytopenic effect of the patient’s plasma when injected into healthy individuals.

5. Variability in the Thrombocytopenic Response of Individual Recipients Following the Administration of the Patient's Plasma

A certain degree of variability in the individual response to the administration of M. K.’s plasma was noted early in the course of this work and confirmed with the increasing number of transfusions in which this plasma was used. Figure 11 illustrates the thrombocytopenic response of various healthy individuals to the administration of a volume of 1.1 ml./Kg. weight of M. K.’s plasma. Many factors appeared to modify the thrombocytopenic response, including the initial platelet level and any fundamental disease present. One patient with acute thrombophlebitis, for example, appeared very resistant to the thrombocytopenic effect of M. K.’s plasma. He received 2.0 ml./Kg. weight of M. K.’s plasma, a dose sufficient to induce a significant and persistent thrombocytopenia in normal recipients. His platelet count fell from 950,000 to 550,000/cu.mm. 32 hours after the administration of plasma but had returned to the original level within 3 hours.

6. Effect of the Injection of M. K.’s Plasma on the circulating Platelets as Observed Directly by the Hamster Pouch Technic*

Male hamsters weighing approximately 100 Gm. each were anesthetized with nembutal intraperitoneally. Seven-tenths ml. of M. K.’s serum per 100 Gm. weight was injected intracardially and a pouch preparation immediately set up by the technic of Lutz et al.37 Microscopic observations at 900 X magnification of the blood vessels of the pouch were started approximately 10 minutes after the plasma had been injected. The vascular wall of the capillaries could be recognized easily. The red cells within the vessel were visually arranged in quickly proceeding columns, arrested intermittently and for a very short time at vascular junctions. The white cells were identified as independent cells crawling along the vessel wall. The most significant feature of this preparation was the presence of small shiny round bodies of hyaline appearance, approximately 7 to 10 microns in diameter, proceeding at high speed along the vessel wall and being intermittently and temporarily arrested at vascular junctions. The number of these bodies increased for the first 10 minutes of observation and then decreased until they had completely disappeared 80 minutes later (90 minutes

* This section was contributed by Dr. Curt Wasastjerna, of Helsinki, Finland, formerly Research Fellow, Blood Research Laboratory, New England Center Hospital, under the auspices of the Rockefeller Foundation.
after the injection of plasma. Direct platelet counts (using Sequestrene-Na2, 1 per cent in sodium chloride solution as diluting fluid) and blood smears were taken at the same time from the hamster's paw. The platelet count dropped from 962,000/cu.mm. to 560,000/cu.mm., 40 minutes after the administration of plasma. Stained blood smears showed few platelet clumps at the beginning of the experiment, but 40 minutes later, large platelet clumps cemented together by a hyaline ground substance were seen. Ninety minutes after the administration of M. K.'s plasma, the platelet count was 888,000/cu.mm. and only a few platelet clumps were visible in smears of the peripheral blood.

Control experiments, injecting human serum from normal donors, were negative. Direct observation of the circulation in the hamster's pouch after direct intracardiac injection of normal human plasma failed to disclose any of the abnormalities just described and caused only a minor and transitory drop in the platelet count.

Finally, antihamster platelet serum was prepared in the rabbit by repeated intravenous injection of isolated hamster platelets. The serum collected, after absorption with hamster's red blood cells to eliminate any antihamster red cell antibody, resulted in the development of an antihamster platelet agglutinin in a titer of 1/32. The intracardiac administration of 0.7 ml./100 Gm. Kg. weight of this serum caused the platelet count to drop from an average of 940,000 to one of 326,000/cu.mm. within 40 minutes. On direct microscopic observation of the hamster's pouch, however, only occasional shiny bodies (presumably agglutinated platelets) were noticed. It is possible that the failure to reproduce the results obtained with the patient's serum may have been due to the low titer of the antihamster platelet serum used.

(d) Experimental Studies in Animals (Rabbits)

M. K.'s blood was collected in 1/5 volume of ACD solution; the plasma separated by centrifugation at 2000 r.p.m. for 30 minutes, transferred to a new container, kept in an ice box at a temperature of 4 C., and used only within twenty-one days of collection. The animals used in the following experiments were albino male rabbits of the average weight of 6 lbs. They were kept in single metallic frame cages, fed water, purina chow and fresh vegetables ad libitum. Each rabbit was kept approximately a week prior to any experiment. During this period of time at least three platelet counts were taken to establish a base line level. M. K.'s plasma was injected through a 23 gauge needle directly into a marginal vein of the ear. Samples of blood for platelet counts were collected before and after the administration of M. K.'s plasma at various intervals of time. Blood was collected directly from the central artery of the ear, avoiding stasis, into a tuberculin syringe containing 3/5 volume of 1 per cent solution of Sequestrene-Na2 in sodium chloride isotonic solution through a 25 gauge needle. The diluted blood was transferred to silicone coated test tubes and within half an hour a direct platelet count was determined by the method previously described. In a number of control experiments, plasma from healthy individuals was injected.

In a number of experiments, rabbits were used which had been subjected to
various surgical procedures,* including splenectomy, laparotomy and ligature of the splenic arteries. The animals were anesthetized with intravenous Nembutal, in a maximum total dose of 18 mg./lb. weight, 10 mg./lb. weight being administered as initial anesthesia and the rest during the course of the operation as required. Sterile precautions were adopted throughout and intramuscular procaine-penicillin (20,000 units/lb. weight) administered daily for the first two days following operation. When the animals were subjected to ligature of the splenic arteries, the “vasa brevia” were left intact to assure sufficient blood flow for the survival of the organ. Following operation, the rabbits were given sufficient time for recovery, approximately five to ten days, after which time the described experiments were conducted.

Figure 15 summarizes the effect of the administration of M. K.’s plasma on the platelet level of normal rabbits, splenectomized rabbits and rabbits in which the splenic arteries had been ligated. In the normal group, the administration of M. K.’s plasma was followed by a prompt drop in the platelet count, with gradual recovery and return to the original platelet level in a period of 4 to 24 hours. Animals in which a “sham” splenectomy had been performed behaved in similar fashion. However, animals in which the spleen had been surgically removed or

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the splenic arteries ligated failed to show any significant drop in the platelet level following the administration of M. K.'s plasma.

Control experiments, in which similar groups of animals were injected with plasma of healthy individuals, were performed. The initial drop in the platelet count was less pronounced and the recovery phase somewhat shorter, but the general pattern of the thrombocytopenic response generally similar. Curiously enough, however, splenectomy and ligation of the splenic arteries failed to prevent the transitory thrombocytopenia due to the administration of normal plasma.

**DISCUSSION**

1. The Platelet Agglutinin (in Vitro Tests)

The main interest of this case lies in the demonstration of a circulating platelet agglutinin and in its in vitro effects. There is no doubt that there are many pitfalls in the demonstration of platelet agglutinins, especially when these are present in low titer. For the most part, false positive tests are due to the frequent occurrence of nonspecific agglutination, providing special precautions are not taken in the preparation and handling of plasma, serum and platelets. In the present case, the agglutinin could be readily and consistently demonstrated in uniformly high titer when citrated plasma or decalcified serum of the patient was tested against platelets from at least 50 donors. The platelet preparations used were various: platelets suspended in their own plasma, isolated platelets resuspended in saline or other fluids, with or without the addition of antiagglutinating agents such as sodium acetate or surface-active agents such as Tween-80 or Triton WR-1339. The agglutination of platelets could be observed directly with the use of phase contrast microscopy immediately following the addition of the patient’s plasma and serum to normal platelet suspensions. The agglutinin was active over a wide range of pH; electrophoretic studies showed it to be a specific protein fraction.*

In a similar case recently reported by Dausset, the antiplatelet agent differed from ours in that the offending factor was lytic. In our own case no evidence of a platelet-lytic effect was present, agglutination being apparently the only mechanism at work. This was demonstrated not only by direct incubation experiments, but also, indirectly, by the absence of any participation of complement in the serum agglutinin-platelet reaction.

Of particular interest were the studies indicating the ability of the platelet agglutinin to inhibit the function of normal platelets. Thus, when the patient's serum was added to normal human platelet rich native plasma, gross clumping of platelets was visible even before clotting took place. Following this procedure, the agglutinated platelets were obviously impaired in their ability to cause clot retraction. Utilization of prothrombin during clotting of the plasma was higher than in control experiments in which the plasma was tested against normal serum or serum from patients with I.T.P., not containing demonstrable platelet agglutinins. Since both clot retraction and prothrombin utilization during clot-

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* The identification, purification and separation of the agglutinin from the serum by electrophoretic technics will be reported elsewhere.
ting are reputed to be either direct (clot retraction) or indirect (prothrombin utilization) functions related to the physiologic integrity of platelets, these findings are paradoxical and require explanation. It may be postulated that the agglutinin was able to injure the platelets in sufficient degree to inhibit clot retraction, simultaneously liberating or activating certain factors which influence the rate and extent of prothrombin utilization during clotting.

2. Is This Agglutinin an Antibody?

Although some reservations may be made as to whether or not the platelet agglutinin demonstrated in our case was an antibody, the weight of the evidence was in favor of this concept. For example, the agglutinin could be removed from the patient’s serum by repeated absorptions with packed platelets (presumably, the antigen); the agglutinin could be eluted from these platelets and was then found to be active in agglutinating fresh normal human platelets; the patient’s platelets, isolated during a transitory elevation of their number following splenectomy, were shown to be “coated” by a substance capable of reacting with anti-human rabbit serum (i.e., positive platelet Coombs test); electrophoretic studies demonstrated a marked increase in abnormal globulin which could be removed, in large measure, by absorption of the serum with normal human platelets. To be sure, the cause for the development of such an auto- and isoplatelet antibody could not be demonstrated, but its effects in vivo, discussed below, suggested that it was an antibody in the broad use of the term. It will be noted that this agglutinin caused striking effects in diminishing the survival of platelets injected into the patient’s circulation or when the patient’s plasma was injected into normal individuals. Thus an “extrinsic” type of platelet destruction, as noted in the auto-immune hemolytic anemia, could be demonstrated. Furthermore, it was possible to study the survival time of the platelet agglutinin when the patient’s plasma was injected into normal individuals. The “half-life” of fourteen days was comparable with that of the anti-Rh0 (D) antibody of erythroblastosis fetalis and of the antibodies detected in the few cases of acquired hemolytic anemia in which such studies have been conducted.

3. In Vivo Tests

(a) Survival of Platelets Injected in the Circulation of the Patient

The results of the experiments “in vitro” could not be taken as a complete indication of the mechanism of the thrombocytopenia in our patient. They were, however, confirmed by the findings obtained in vivo, both following the administration of platelets to the patient and of her plasma to normal recipients. Our own results and those of others have demonstrated the rapid disappearance of platelets injected into the circulation of patients with I.T.P. The disappearance time varied greatly and appeared to be related to such factors as the original platelet level, the size of the spleen, the presence of active bleeding, and, above all, the type of case, whether acute or chronic. In all cases of I.T.P., however, the rate of disappearance of platelets from the circulation was much more rapid than that observed in cases of secondary or “amegakaryocytic” thrombocytopenia. The present case revealed an exaggerated picture of this
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type of reaction. The administration of platelet rich polycythemic blood by direct transfusion elevated the platelet count only while blood was being administered. At the end of the transfusion, the platelet count was actually lower than the original one, a finding frequently observed when normal plasma was administered to normal recipients, but not when platelet rich blood was given. When an isolated platelet suspension was administered, not even a temporary rise of the platelet count was observed. These results were essentially similar before and after splenectomy. The presence of a circulating platelet agglutinin in the patient might have been directly responsible for the drastically shortened survival of injected platelets.

(b) Effect of the Administration of the Patient’s Plasma into Normals

(A) On the platelets. The administration of the patient’s plasma into normal recipients was also followed by a series of striking effects, amongst which extreme thrombocytopenia and clinical purpura were outstanding. In our experience of 35 cases with the injection of plasma of patients with I.T.P. into normals, this finding was quite exceptional, in that, although a relatively mild thrombocytopenia usually followed the administration of plasma to normal recipients, a marked reduction took place in only one other case. It was possible to work out a dose of the patient’s plasma that would induce significant changes in number and appearance of platelets, and in the appearance of megakaryocytes in the recipient, without causing marked thrombocytopenia and purpura.

(B) Platelet morphology following the administration of the patient’s plasma to normal recipients. The morphologic abnormalities of the platelets seen for a few days in the circulation of the recipient have already been described and it may be added that they were indistinguishable from those of the platelets of M. K. Since the time of Wright, “macroplatelets” with various degrees of staining abnormalities have been observed in periods of active platelet regeneration. It is customarily conceded that they “either represent forms fragmented from areas close to the nucleus after most of the cytoplasm has been stripped of fully differentiated material or come from incompletely differentiated megakaryocytes (megakaryoblasts, lymphoid megakaryocytes)” (Tocantins). Similarly in our case it could be postulated that the platelets observed both in the patient and in the recipient receiving her plasma represented either young platelets or platelets released as such by young and abnormal megakaryocytes. Against this interpretation was the appearance of almost total lack of platelet formation from any of the megakaryocytes, immature and mature, at the time when these abnormal platelets could be found in the peripheral circulation. Also, the abnormal platelets persisted long after the megakaryocytes of the recipient had resumed a normal appearance.

An alternative explanation may be offered, namely that these abnormal, giant platelets represented, in fact, platelets acted upon by the abnormal agglutinin. This interpretation is favored by the demonstration that platelets were, in effect, coated with protein material reacting with antihuman globulin rabbit serum. It was also observed that the presence of abnormal platelets was in direct time relationship with the persistence in the circulation of the recipient of the transmitted anti-platelet agglutinin. This was demonstrable for a period
of twelve to fourteen days; the number of abnormal platelets returned to normal in approximately the same period of time. Against this interpretation is the failure to reproduce comparable morphologic alterations in vitro when normal platelets were incubated with the patient’s plasma and serum. When one sums up the available evidence, the most acceptable explanation seems to be that these platelets represent elements produced and released by abnormal, immature megakaryocytes. If this explanation is correct, it should again emphasize that the presence of a circulating platelet agglutinin may be responsible for the morphologic and developmental alterations in the megakaryocytes, to be presently discussed.

(C) Changes in morphologic characteristics of the megakaryocytes following administration of the patient’s plasma in normal recipients. As already described, the administration of M. K.’s plasma into normal recipients was followed by the appearance of marked qualitative and functional alterations of these cells. To summarize, they consisted of: (1) reduction and, often, complete loss of granularity of the cell; (2) intense vacuolization and degeneration of the cytoplasm; (3) loss of platelet formation from the periphery of the cell; and (4) coarsening of the chromatin and development of other apparently degenerative changes in the nucleus. These alterations were most evident between 2 and 24 hours after the administration of the M. K. plasma and lasted four to five days. From the sequence of events, as followed in bone marrow aspirates taken at regular intervals, it is probable that the injection of the agglutinin resulted in an immediate attack on the platelets at the surface of the megakaryocytes and those ready to leave the cell, followed by an attack on the megakaryocyte itself. Thus the appearance of the megakaryocytes in the recipient at the peak of the induced thrombocytopenia was similar to that in the patient M. K. herself.

In the patient, splenectomy failed to induce any evident change in the morphology of the megakaryocytes. Similarly, the administration of M. K.’s plasma to splenectomized recipients caused identical alterations of the megakaryocytes. This seems to indicate that, whatever effect the agglutinin had on the megakaryocytes, it was not mediated through the spleen. It is also possible that the abnormal agglutinin influenced the normal process of maturation of the megakaryocytic cells either directly or by destroying the older cells. In the patient, as well as in the recipients of her plasma, there was an increased number of immature megakaryocytes (promegakaryocytes, intermediate megakaryocytes), as well as lymphoid megakaryocytes. It may also be thought that this finding represented a more active megakaryocytic proliferation in the bone marrow due to the increased demand for platelets in the peripheral circulation, where they were quickly disposed of. Such megakaryocytic findings, it should be remembered, are observed in most cases of I.T.P. The gross morphologic alterations of the megakaryocytes appear to suggest, however, that the effect of the agglutinin consisted mainly in a direct damage of these cells.

Thus, at least three independent mechanisms for the patient’s thrombocytopenia were suggested by the results of the various in vivo tests: (1) a direct attack upon the circulating platelets, causing their rapid disappearance from the circulation; (2) a direct attack on the platelets at the surface of the megakaryocyte and (3) an attack on the megakaryocyte itself. A subsidiary phe-
nomenon was the possible development of abnormal platelet morphology and presumably of abnormal function of the surviving platelets. If this is the case, it would probably explain why the bleeding time remained extremely prolonged after splenectomy (although the tourniquet test became normal) even when the platelet count became temporarily normal. A functional abnormality of platelets may reflect itself either as a total failure or as a separate insufficiency of one or more hemostatic functions in which platelets are believed to take part: clot retraction, degree of capillary fragility, bleeding time and prothrombin utilization during clotting. The future will show whether abnormalities in chemical constitution of platelets are responsible for these possibly individual failures of the various platelet functions. The extreme hypothesis might be made that, of the various components of platelets responsible for their various functions, the one involved in the control of the bleeding time was irreversibly abnormal in this patient.

(D) Attempts to modify the activity of the platelet agglutinin. A number of interesting findings were obtained in various attempts to reduce the platelet agglutinin titer of the patient's serum and its activity in the various in vivo tests. As previously described, administration of ACTH and cortisone before and after splenectomy alike, resulted in minor reduction of the titer of the agglutinin, but failed either to elevate the patient's platelet count to any appreciable extent or to cause reduction of the ability of her plasma to induce thrombocytopenia in normal recipients. It is generally agreed that prolonged administration of corticotropin or cortisone reduces the rate and amount of antibody produced. In view of the extremely high titer of the agglutinin, which made the appreciation of minor or moderate differences difficult to evaluate, this finding probably does not militate against the antibody nature of the agglutinin. Splenectomy, which had a rather complicated effect on the various hemostatic functions of the patient, did not appreciably affect the titer of the agglutinin, thus seemingly excluding the role of the organ in the production of this agent, although the platelet count became quite high following operation. Only repeated venesections and replacement with normal blood, with the physical depletion of the amount of circulating agglutinin, had appreciable effects on the agglutinin titer of the patient's serum, even though the platelet count was only slightly affected.

4. The Role of the Spleen

(a) Results of Splenectomy

The possible role of the spleen in the pathogenesis of the thrombocytopenia of this patient and the effects of splenectomy are of unusual interest. As mentioned before, splenectomy was followed by the following significant changes: (1) complete arrest of the spontaneous bleeding manifestations, with normalization of the capillary fragility, although the bleeding time remained persistently prolonged; (2) temporary elevation of the platelet count, with final adjustment to a subnormal level, but higher than the original one; (3) very fleeting reduction of the agglutinin titer, with prompt return to the original value after a few days. These effects were accompanied by failure to modify the thrombocytopenic effect of the patient's plasma when administered to normal recipients. The temporary rise in the platelet count was reasonably striking (reaching a value
of 600,000/cu.mm., but might have been nonspecific, since it was not
maintained.

There can be no question but that a temporary elevation of the platelet count
to normal levels in patients with I.T.P. may be unrelated to splenectomy. The initial reduction in the titer of the circulating agglutinin for a few days
following splenectomy indicated that the spleen might conceivably be the organ
producing the antibody and that splenectomy would ultimately result in complete
recovery. The subsequent return of the agglutinin to the original "in vitro"
level and the failure of splenectomy to affect the ability of the patient's plasma
to cause thrombocytopenia in normal recipients, conclusively proved that the
spleen could not be considered the only or primary source of production of the
agglutinin. In view of the extremely high titer of antibody, one cannot exclude
the possibility that splenectomy might have been followed by some reduction of
agglutinin production not readily demonstrable with the rather insensitive
techniques available at the present time.

(b) Experiments with Induced Thrombocytopenia in Splenectomized Recipients
and Experimental Animals

In evaluating the pathogenesis of the thrombocytopenia in our patient, a
number of observed facts should be kept in mind: (1) the relatively moderate
thrombocytopenic response to injected M. K. plasma of splenectomized
individuals when compared to that of normal individuals; (2) the ability of splec
sectomized animals or of animals in which the splenic arteries had been ligated to
resist the thrombocytopenic activity of our patient's plasma; (3) the moderate
rise in the platelet count of our patient following splenectomy. As previously
described, administration of the M. K. plasma, which induced thrombocytopenia
in normal rabbits when given at rather high doses, failed to do so when the plasma
was given to rabbits which had been previously splenectomized or in which the
splenic arteries had been ligated. This finding may be related to the results previ-
ously reported by Bedson and by Elliott et al., who showed that splenectomy
greatly suppressed the thrombocytopenia due to the administration of anti
rabbit-platelet serum. We confirmed these findings and the same preventive
effect was observed after ligation of the splenic arteries. These experiments may,
of course, be of significance but their implications should not be overempha-
sized for a number of reasons. The amount of M. K.'s plasma administered to
rabbits was much larger in terms of body weight than that administered to
humans and yet the thrombocytopenic effect was much smaller than that ob-
served in humans. Secondly, when rabbits were killed at the peak of the throm-
bocytopenia induced by the intravenous administration of M. K.'s plasma, the
blood collected from the spleen and sections of the organ failed to disclose any
excessive number of platelets in the splenic blood, sinuses and pulp. Finally, the
significance of experiments in which animals receive heterologous blood should
always be questioned, especially if not in accord with results obtained by homol-
ogous species experiments.

With these limitations in mind, it appears that the spleen did not influence
the disposal of platelets injected into M. K.'s circulation, since their disappear-
ance was equally fast prior to and following splenectomy. To a small extent, it
might have influenced the degree of thrombocytopenia following the adminis-
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We have been able to oxidize the M. K.'s plasma into normal humans and experimental animals. This conclusion may be of significance, since it might help to clarify the relationship of the various pathogenetic mechanisms in I.T.P. As outlined in a recent article from this laboratory, the thrombocytopenia of I.T.P. may be explained by one or more of the following hypotheses: (1) increased utilization of platelets in the peripheral circulation; (2) destruction of platelets in the peripheral bed, possibly through the activity of circulating antiplatelet substances; (3) "sensitization" of platelets by circulating agents and consequent trapping and destruction of these platelets in the spleen or other reticulo-endothelial stations; (4) direct trapping and destruction of normal platelets by an abnormal spleen. Our recent work with direct experiments has brought us to minimize the role of the spleen as a selective trapping or lytic organ with respect to platelets, since when normal platelets were injected into the circulation of patients with I.T.P. (of the "acute" type, in our experiments), the spleen did not appear to possess any particular sequestrative activity as compared to other organs. This, probably was not the case in our patient, M. K., where the circulating platelet agglutinin probably had the opportunity of "sensitizing" platelets, thus making them more prone to destruction in various reticulo-endothelial organs, of which the spleen is probably the most important.

(c) General Interpretation

The mechanism of the thrombocytopenia in this patient exemplifies, we believe, the concept that I.T.P. is an extremely protean disease, in which many different mechanisms are at play, singly or together, in a given case. This diversity of pathogenetic mechanism may even reflect itself in definite clinical patterns; thus the differentiation between "acute" and "chronic" cases of I.T.P. has been recently emphasized from this laboratory. It cannot be denied that this distinction is by no means sharp in many cases. However, it serves a useful purpose in directing the treatment of the individual case and may prove to be based on significant differences in the basic pathogenetic mechanism. In the acute cases, we have thus far been unable to demonstrate any circulating agglutinating anti-platelet agent, at least by in vitro tests. When one observes the bone marrow of these patients, the great prevalence of immature megakaryocytes is striking and gives the impression that in these cases a sudden, acute block may be present which inhibits normal megakaryocytic maturation and formation and release of platelets. The number of eosinophiles and lymphocytes may also be increased and, in many instances, an allergic or hyperimmune mechanism has been incriminated. In the "chronic" cases, the alterations of the bone marrow megakaryocytes are equally marked (although one gains, at times, the impression that the maturation arrest is not so pronounced as in the "acute" cases). Moreover, it is possible to demonstrate in some patients the presence of circulating antiplatelet agents, with a probable destructive effect on the circulating platelets. Finally, in the "symptomatic" cases, the spleen presents a primary disease (disseminated lupus, tuberculosis, sarcoidosis, etc.); no platelet destructive agents can be demonstrated in the circulation of these patients, but the alterations of the megakaryocytes of the bone marrow are indistinguishable from those of the "acute" and "chronic" types of I.T.P.

When these various factors are put together in an attempt to present a unified
explanation, the difficulties at once become obvious. There can be no doubt that the thrombocytopenia of I.T.P. represents a disequilibrium between platelet formation and platelet destruction. The uniform abnormality of platelet formation seems documented by the morphologic alterations observed in the megakaryocytes in all types of the disease. This defect of platelet formation may well be fundamental and, at least in many cases, undoubtedly exaggerates the activity of any mechanism of peripheral destruction of platelets which has been shown to exist in most cases of I.T.P. by the results of platelet transfusion experiments. The inhibition of formation and release of platelets from the megakaryocytes in I.T.P. may well be due to splenic activity. Only in few cases, like our own, is it possible to demonstrate a circulating anti-platelet agent, directly affecting the platelet-producing activity of megakaryocytes, as well as affecting the platelets directly. In such cases, the spleen, as possibly other reticulo-endothelial organs, may be able to dispose of these platelets, "sensitized" by the action of the circulating anti-platelet agent.

The experiments in rabbits, which seem to contradict the results obtained in our human in vivo studies, must be considered as quite preliminary in nature and are thus to be accepted with considerable caution. The agglutinin used was an antihuman one and results obtained with such a heterologous agent might be considered quite artificial and the animals received extremely large doses of the agglutinin. If the experiments are at all significant, however, they would seem to indicate a subsidiary mechanism through which the spleen might, in cases where a high potency anti-platelet agent is present in the circulation, dispose quickly of platelets "sensitized" by this agent. In this function, the spleen may act either specifically or like one large area of the reticulo-endothelial system. Further experimental work on these points is certainly desirable.

**SUMMARY**

1. A case of chronic idiopathic thrombocytopenic purpura (I.T.P.) exhibited a circulating platelet agglutination of high titer. This could be demonstrated not only through its ability to clump various platelet preparations at extremely high titers, but also to interfere with the functional activity of normal platelets. The agglutinin could be absorbed upon packed platelets and, when eluted from them, its agglutinating activity remained intact. Platelets obtained from the patient were found to be "coated" by an agent capable of reacting with antihuman globulin rabbit serum. Complement played no role in the reaction of agglutinin and platelets. Various properties of the agglutinin were established and this was also characterized and purified by electrophoretic techniques. The agglutinin was found to be in the $\gamma_2$ globulin area and to represent 9.33 per cent of the entire serum protein. All indications pointed to the characterization of the agglutinin as a platelet iso- (and auto-) antibody, although this could not be definitely proven.

2. Platelets injected into the circulation of the patient disappeared very promptly. When the patient's plasma was injected into normal recipients, a series of effects followed: (a) striking degenerative changes of the bone marrow megakaryocytes with lack of formation of platelets; (b) an extreme degree of platelet reduction with the development of hemorrhagic phenomena; (c) detectable platelet agglutinin in the recipient's serum persisting for twelve to four-
ten days. The recipient platelets were also found to be coated with a substance capable of reacting with antihuman globulin rabbit serum (positive Coombs test).

3. Various procedures including the administration of cortisone and splenectomy failed to modify the thrombocytopenic response of normal recipients to the patient’s plasma, although appreciable individual variations in thrombocytopenic response were observed. Repeated venesections, however, resulted in a definite reduction in the concentration of platelet agglutinin. The titer of agglutinin in the patient remained unmodified after splenectomy.

4. Splenectomy was followed by a complete arrest of the bleeding manifestations and a temporary rise in the platelet count, which soon fell to a relatively low level, although somewhat higher than that prior to splenectomy. Prothrombin utilization during clotting and capillary fragility slowly returned to normal. On the other hand, the appearance of the platelets in the peripheral blood and of the bone marrow megakaryocytes remained unmodified, and the bleeding time remained prolonged.

5. The response of splenectomized recipients to the patient’s plasma was of the same immediate intensity, but of much shorter duration than that of normal recipients. Since, furthermore, splenectomy induced a moderate rise in the patient’s platelet count, but failed to reduce the concentration of the serum platelet agglutinin, it is postulated that, in this particular case, the thrombocytopenia was probably due to the direct injury of circulating platelets and of the bone marrow megakaryocytes by the circulating agglutinin, thus resulting not only in increased destruction but in reduced formation and release of platelets. Some of our experimental results in animals also indicate the possibility of removal of “sensitized” injured platelets by the intact spleen.

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Studies on Platelets: IX. Observations on the Properties and Mechanism of Action of a Potent Platelet Agglutinin Detected in the Serum of a Patient with Idiopathic Thrombocytopenic Purpura (with a Note on the Pathogenesis of the Disease)

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