

## Studies on Thrombopoiesis

### II. Thrombocytopoiesis in Vitro from the Bone Marrow of Patients with Idiopathic Thrombocytopenic Purpura

By G. IZAK AND D. NELKEN

With the technical assistance of MISS A. HERZOG

**P**REVIOUS STUDIES of thrombocytopoiesis in tissue culture of normal human and animal bone marrow<sup>1</sup> showed that megakaryocytes produce a large number of viable platelets within 24 to 60 hours.

In the present paper observations are reported on thrombocytopoiesis from megakaryocytes of three patients suffering from idiopathic thrombocytopenic purpura (I.T.P.). In addition, studies were made of thrombocytopoiesis in cultures of nine normal bone marrows incubated with anti-platelet sera (A.P.S.)

#### MATERIAL AND METHODS

Three patients, aged 19, 24 and 46, suffering from I.T.P. and nine individuals aged 23–39, with no hematologic disorders were studied. The diagnosis of I.T.P. was established according to the usual criteria and relevant hematologic findings are summarized in table 1.

The culture media and the technic employed have been described elsewhere.<sup>1</sup> Bone marrow samples were obtained by aspiration from the sternum.

The bone marrow samples taken from the nine individuals with no hematologic disorders were each divided into two equal parts. One portion was dealt with as described elsewhere,<sup>1</sup> while to the other half, 0.1 ml. anti-platelet serum was added just prior to explantation. The further steps taken were also identical with those described elsewhere.

Anti-platelet serum was prepared as follows: Patients suffering from I.T.P. with a high titer of anti-platelet antibodies in their serum were bled under sterile conditions; the serum was activated and stored at  $-20^{\circ}\text{C}$ . until used. Thromboagglutinins were elevated by incubating 0 platelets with doubling dilutions of these sera for 6 hours at  $33^{\circ}\text{C}$ . The last tube to show microscopic agglutination ( $\times 100$ ) of platelets was taken as the titer.

Two anti-platelet sera were used, one from a patient of blood group AB and the other from a patient of group A. In the latter case, the serum was absorbed with erythrocytes of group B before use.

#### RESULTS

##### *In I.T.P.*

Direct observations of thrombocytopoiesis in all three patients gave similar results and these changes will be reported together.

The number of megakaryocytes in these bone marrow samples appeared much greater than in the normal marrows previously observed. The cells seen were of various sizes, most of them round, and in none could degenerative changes be observed.

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From the Department of Medicine/B, Hadassah University Hospital, and the Department of Clinical Microbiology, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

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TABLE 1.—*Findings Relevant to Diagnosis of I.T.P. in Three Patients*

No.	Age	Sex	Plate-lets	Bleed- ing time <sup>14</sup>	Clotting time <sup>15</sup>	Clot reaction <sup>16</sup>	Plasma prothrombin		Serum prothrombin		S.P.C.A. <sup>20</sup> %	Fibrinogen <sup>19</sup> mg%	Tourniquet test	Platelet agglutinins
			cu.mm.				Quick <sup>17</sup> %	Soulier <sup>18</sup> %	Quick <sup>17</sup> %	Soulier <sup>18</sup> %				
1	24	M	21,000	18'	6.30'/8'	Incom- plete after 24 hours	100	90	75	25	107	290	++	+++
2	19	F	27,000	17'	5.5' /7.5'	Incom- plete after 24 hours	100	100	100	80	0	380	+++	-
3	46	F	18,000	15'	4.3' /7.0'	Incom- plete after 24 hours	100	100	65	30	75	270	+++	+

\* S.P.C.A. = Serum Prothrombin Conversion Accelerator.

The process of thrombocytopoiesis was essentially similar in these samples to that observed in normal bone marrows.<sup>1</sup> However the appearance of coarse granules, their aggregation into irregular, massive cords and subsequent breakdown into platelets seemed to progress much more rapidly than in the normal bone marrow specimens (figs. 1-6). In some instances the process in all cells seen was completed within 12 to 16 hours as against the 48 to 60 hours required in normal bone marrow samples. The number of "inactive" cells was similar to that seen in normals. The other findings, such as the crossing of megakaryocytes by leukocytes and the finding of red blood cells in the cytoplasm of megakaryocytes were also similar to the controls.<sup>1</sup>

The striking difference between normal bone marrow culture and that of I.T.P. patients came to light when the daily separation of platelets revealed a steady decrease in their number and a corresponding decrease in their serotonin\* binding capacity, which persisted throughout the whole period of observation (fig. 7). Furthermore degenerative changes in the platelets, such as swelling and concentration of the granules in one portion of the hyalomer, were frequently seen amongst the thrombocytes after their separation from the rest of the bone marrow tissue. During the direct observation of this process the impression was gained that actively moving cells actually phagocytosed large numbers of platelets. Isolated platelets were scarcely encountered in the culture but a few masses of agglutinated thrombocytes were seen (fig. 6, 2).

#### *Human Bone Marrow Cultured Together with A.P.S.*

The process of thrombocytopoiesis was similar to that observed in bone marrow cultures from patients with I.T.P. The speed with which the cytoplasm of megakaryocytes transformed into masses of platelets was much greater than in any of our previous experiments. Within 4 to 5 hours almost all megakar-

\* Chemical characterization of the serotonin was not performed and there is thus a possibility that a "serotonin like substance" was released from the platelets and measured. It is assumed however, in the present paper, that the substance measured was, in fact, serotonin, and it is reported as such.

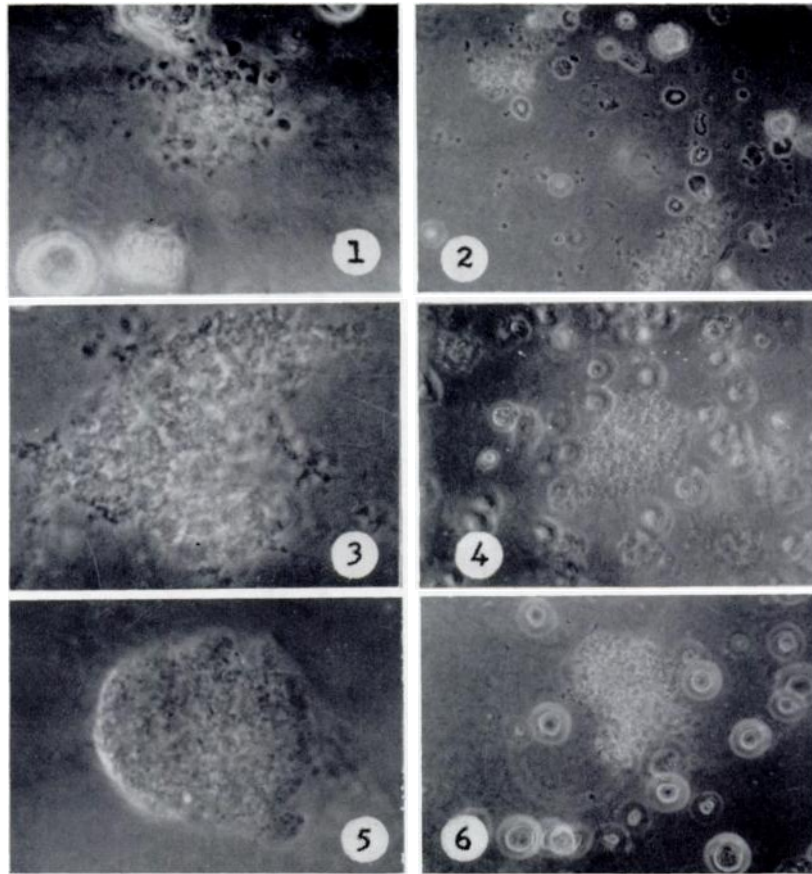


FIG. 1.—Megakaryocyte showing almost complete breakdown into platelets 6 hours following its explantation. ( $\times 1300$ .)

FIG. 2.—Platelets in a 17 hours culture; most of the thrombocytes are concentrated into two clumps, a few isolated ones can be seen. ( $\times 650$ .)

FIG. 3.—Megakaryocyte showing pseudopodia formation. The peripheral portion of the pseudopodia have broken down into platelets, whereas the portion toward the center of the cell is filled with coarse strongly refractile bands. These changes occurred 7 hours following the explantation. ( $\times 1300$ .)

FIG. 4.—Relatively small megakaryocyte filled with coarse granules. These changes together with the disappearance of the cytoplasmic and nuclear border occurred 6 hours following the explantation. ( $\times 650$ .)

FIG. 5.—The beginning of pseudopodia formation at both the lower and upper part of a megakaryocyte after 6 hours of incubation. ( $\times 2000$ .)

FIG. 6.—Platelet clumping observed 16 hours following explantation. ( $\times 650$ .)

yocytes broke down and the platelets thus produced were seen in the form of clumps. Almost no "inactive" megakaryocyte could be found.

When the incubated samples were removed daily and the separation of platelets was attempted, the striking decrease in the number and serotonin binding capacity of the platelets was greater than in cases of I.T.P. (fig 8). Most of the platelets examined under the microscope showed signs of degeneration. Here again the impression was gained that many platelets underwent phagocytosis.

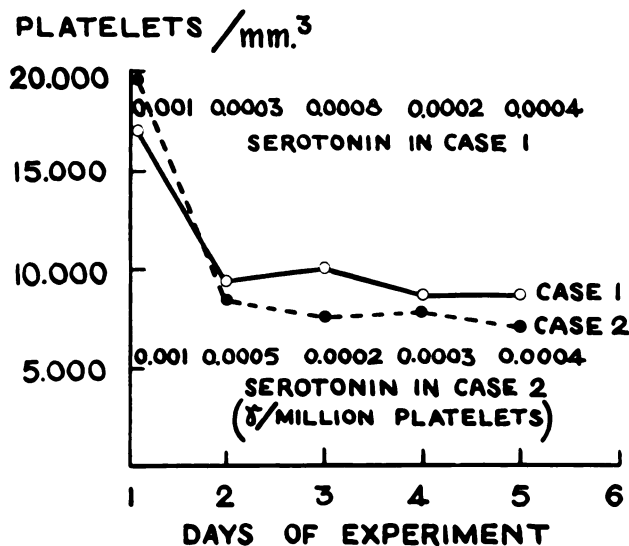


FIG. 7.—Two cases of idiopathic thrombocytopenic purpura: The platelet count decreases gradually with a drop in the serotonin absorbing capacity.

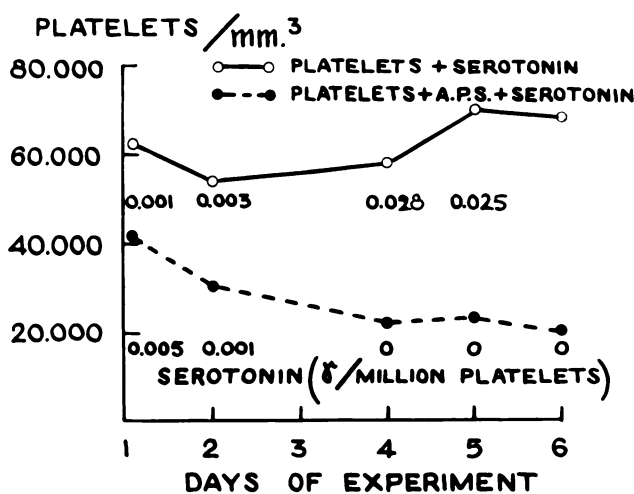


FIG. 8.—Upper curve: normal bone marrow culture. The platelet count increases parallel to the rise in the serotonin absorbing capacity. Lower curve: following the addition of anti-platelet serum to the same bone marrow culture, the platelet count drops together with the serotonin absorbing capacity.

Despite the direct observation of platelet-phagocytosis the following experiment was devised in order to furnish more objective evidence of the actual presence of this phenomenon in our experiments.

One hundred ml. of blood was withdrawn from the cubital vein of healthy individuals and clotting was prevented by heparin. The blood was centrifuged at 500 r.p.m. for 5-7 minutes and the plasma was removed and re-centrifuged at 2000 r.p.m. for 10 minutes. The platelets thus obtained were washed three

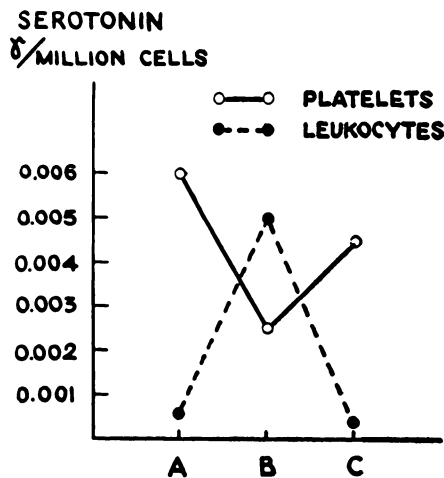


FIG. 9a

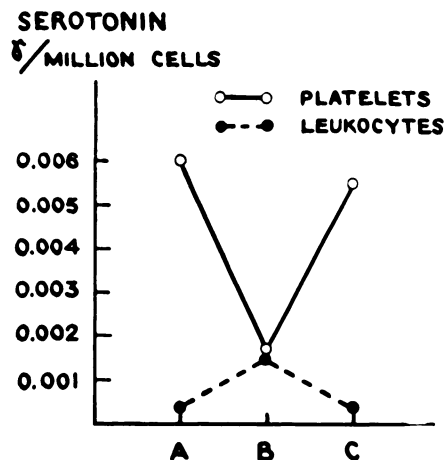


FIG. 9b

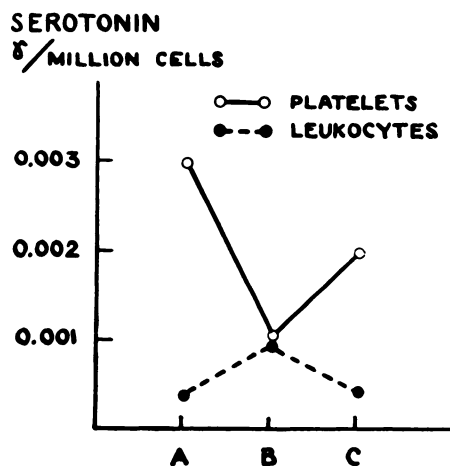


FIG. 9c

FIG. 9a, b, c

A.—Serotonin content of platelets and leukocytes incubated separately.

B.—Serotonin content of platelets treated with anti-platelet serum, incubated together with leukocytes, separated and washed.

C.—Serotonin content of platelets and leukocytes incubated together, separated and washed. (Straight line—platelets; broken line—leukocytes.)

times in Tyrode's solution, resuspended in 2 ml. of Tyrode, counted and examined with a phase microscope.

The buffy coat layer of the sedimented cells was removed, resuspended in Tyrode solution and centrifuged at 500 r.p.m. for 5-7 minutes. The sediment was washed three times in Tyrode's solution, centrifuged and resuspended in 2 ml. of Tyrode solution. The suspension thus obtained consisted mainly of white blood cells with a few red cells and platelets. The leukocytes in the suspension were counted.

0.5 ml. of the leukocyte suspension was then mixed with 0.5 ml. of platelet suspension and incubated for 60 minutes at 37 C., after which the mixture was centrifuged at 500 r.p.m. for 5 minutes. The supernatant was removed, centrifuged for 10 minutes at 2000 r.p.m. and the platelets thus sedimented were washed three times in Tyrode's solution. They were resuspended, counted, frozen and thawed several times and their serotonin content determined. The remaining leukocytes were also washed three times, shaken vigorously with glass beads for 15 minutes and the serotonin determined in the supernatant. The same procedure was repeated with two additional samples, following the addition of 0.02 ml. anti-platelet serum to the platelet suspension 5 minutes prior to its mixing with the white blood cell suspension. As a control the serotonin content of 0.5 ml. of platelet suspension and 0.5 ml. of leukocyte suspension was determined following their incubation at 37 C. for 60 minutes.

While platelets incubated with leukocytes and subsequently separated retained serotonin corresponding in amount to that found in the control platelet suspension, thrombocytes treated with A.P.S. lost most of their serotonin content which could be recovered in the supernatant fluid (fig. 9a, b, c). In addition, while leukocytes incubated with platelets contained almost no serotonin after separation and washing, they were found to contain large amounts of serotonin after incubation with A.P.S. treated thrombocytes (fig. 9a, b, c). As leukocytes are unable to absorb serotonin per se (personal observation) these results indicate that the leukocytes must have absorbed serotonin by taking up serotonin-containing platelets.

#### DISCUSSION

Frank<sup>2, 3</sup> and Dameshek and Miller<sup>4</sup> have reported morphologic changes in the megakaryocytes of patients with I.T.P.; these changes being different in acute I.T.P. from those seen in the chronic type of the disease.<sup>5</sup> Kissmeyer-Nielsen reported an increase in the number of marrow megakaryocytes in both acute and chronic I.T.P., with evidence pointing to diminished platelet production.<sup>16</sup> Frank,<sup>2, 3</sup> Dameshek and Miller<sup>4</sup> attributed these changes to an inhibiting agent liberated by the spleens of patients with I.T.P. and the occasional prompt disappearance of these changes following splenectomy was offered as evidence in support of this view. Other authors considered thrombocytopenia to be a result of destruction of platelets in the spleen by lysis<sup>7</sup> or phagocytosis<sup>8</sup> as indicated by the short survival of transfused normal platelets.<sup>9, 10</sup> Both these views were supported by experimental evidence as a result of which Moulten concluded that the spleen contains at least two factors: thrombocytosin, which is said to stimulate, and thrombocytopenin, to depress the production and delivery of platelets from megakaryocytes.<sup>11</sup> More recently the finding of platelet agglutinins in patients with chronic I.T.P.<sup>12, 13</sup> directed attention to the role of an "immunoallergic" mechanism in the pathogenesis of the disease. On the basis of a large number and variety of observations, Stefanini and Dameshek<sup>5</sup> stressed the variability of the pathogenic mechanisms in different types of I.T.P. The same authors further concluded that a combination of these pathogenic

factors could explain all manifestations of the disease in the peripheral blood and bone marrow, be they of the acute or of the chronic type.

In the present study a large number of living megakaryocytes were observed from patients with chronic I.T.P. in two of whom platelet agglutinins could be demonstrated (the serum of the third patient was not tested). These cells were similar in microscopic appearance to those isolated from bone marrow of normal healthy individuals.<sup>1</sup> The break-down of these megakaryocytes to platelets however, proceeded more rapidly than in normals and the process was usually completed within 12 to 24 hours. The mode of platelet production from stage to stage was identical with that observed in normal bone marrows. The proportion of actively producing megakaryocytes and those which remained stationary throughout the observation was also similar to that seen in normal bone marrow cultures. The striking difference between the thrombocytopoiesis observed in these cultures and those of normal individuals was that platelets, once they separated from the cytoplasm of the megakaryocytes, remained clumped together and both clumps and the few individual platelets present seemed to undergo phagocytosis by the myeloid elements and the reticulum cells present. The platelets separated from these cultures showed marked degenerative changes, a gradual drop in number and a proportional decrease in their serotonin absorbing capacity.

These changes indicate that the plasma added to the culture medium caused, by its platelet agglutinin content, degenerative changes in the platelets and their agglutination and probably aided their incorporation and final destruction by the phagocytes. There was no evidence however of injury to the megakaryocytes either in their morphologic appearance or in their platelet producing ability. Whether the acceleration of platelet production could be considered as evidence of damage to the megakaryocytes cannot be decided. In no instance however, was there evidence of suppression of production of platelets under the experimental conditions described.

It is of interest that the sequence of events observed during thrombocytopoiesis of patients with I.T.P. could be reproduced in normal bone marrow cultures by the addition of a potent A.P.S. The only difference was that the process proceeded at an even faster rate in cultures with A.P.S. than in those of I.T.P. patients. This fact furnishes further evidence of the importance of platelet agglutinins in the pathogenesis of I.T.P.

That platelet phagocytosis must have taken place in these cultures is indicated by the results obtained when A.P.S. treated platelets underwent phagocytosis when incubated with polymorphonuclear leukocytes. As it was found that the leukocyte is not capable of absorbing serotonin, the marked increase in serotonin content following incubation was attributed to the incorporation of thrombocytes into the cells by phagocytosis.

The observations reported here were made in tissue cultures, an artificial set-up, which does not permit definite conclusions as to the identity of the process in the bone marrow of patients with I.T.P. Taking into consideration that the pattern of changes fits the general pattern of the pathogenesis of I.T.P., it would seem probable that the conclusions arrived at are applicable to the conditions pertaining *in vivo*.

## SUMMARY

Thrombocytopoiesis was studied by direct, continuous observation in tissue cultures of bone marrows taken from three patients with chronic idiopathic thrombocytopenic purpura and from normals following the addition of a potent anti-platelet serum to the culture media.

The process of platelet production was similar in these two conditions and followed the pattern observed in bone marrow cultures of healthy individuals. The breakdown of megakaryocytes of patients with idiopathic thrombocytopenic purpura and those treated with anti-platelet serum was greatly accelerated.

No morphologic evidence of injury to the megakaryocytes was present.

The platelets produced showed degenerative changes, they were agglutinated and underwent phagocytosis by the myeloid elements and reticulum cells were present in the cultures.

## SUMMARIO IN INTERLINGUA

Thrombocytopoiese esseva studiate per medio de directe e continue observationes in histoculturas de medulla ossee prendite ab tres patientes qui habeva chronic idiopathic purpura thrombocytopenic, e etiam in culturas de medulla ossee normal al quales un potente sero anti plachettas habeva essite addite.

Le processo del production de plachettas esseva simile sub iste duo conditiones e sequeva le curso observate in culturas de medulla ossee prendite ab individuos de bon sanitate. Le destruction de megacaryocytos ab patientes con idiopathic purpura thrombocytopenic, assi como de megacaryocytos tractate con sero anti plachettas, esseva multo accelerate.

Nulle indicationes de insultos al megacaryocytos esseva presente.

Le plachettas producite manifestava cambios degenerative; illos esseva agglutinate; e illos suffreva phagocytose per elementos myeloide e per cellulas reticular trovate in le culturas.

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