Studies on Thrombopoiesis

I. Thrombocytopoiesis in Vitro: Experiments with Animal and Normal Human Material

By G. Izak, D. Nelken and J. Gurevitch

With the technical assistance of Miss A. Herzog

The use of phase- and electron microscopy has led to a considerable increase of knowledge on the morphology of the platelets, and a number of detailed reports dealing with biochemical aspects of the platelets in health and disease have recently appeared.

It is now generally accepted that the site of origin of the platelets is the megakaryocyte and several attempts have been made to classify these cells according to their stage of development with regard to platelet production. Such classifications are based on morphologic features seen in the stained bone marrow film, or on the quantitative relationship to circulating blood platelets.

The present study deals with continuous direct observations on platelet production in tissue cultures of bone marrow from guinea pigs, mice, dogs and normal adult human beings.

Material and Methods

The bone marrow of six men and five women free of hematologic disorders were examined. Sixteen guinea pigs weighing between 200 and 450 grams, four dogs weighing between 7 and 12 kilograms and 5 mice, were included in this study. From each bone marrow sample, ten to fourteen slide cultures were prepared for direct observation, and in over half quantitative determination of thrombocyte production was made in Carrel flasks.

Except for the slides and cover slips used in the tissue cultures, siliconized glassware was used throughout; all experimental procedures were performed under sterile conditions.

Bone Marrow. Bone marrow was obtained from the proximal epiphysis of the femur of mice, guinea pigs and dogs. Large amounts of bone marrow were obtained from dogs by sucking Tyrode solution through their ribs with the aid of a vacuum. Human marrow was obtained by sternal aspiration. The material obtained was halved. One portion was suspended in Tyrode solution containing Penicillin 80 units and Streptomycin 100 μg per ml., while the remainder was spread on a sterile filter paper and the tissue fragments were directly explanted into the culture medium (see below). The bone marrow suspension was centrifuged at 3000 r.p.m. for twenty minutes when the middle of the three layers obtained, contained a large number of megakaryocytes, as well as large, mainly myeloid elements. Tiny portions of this layer consisting of a thick suspension of cells, were explanted into the culture medium.

Tissue Cultures. A 3.0 x 2.5 cm. cover slip was fastened with paraffin wax to an aluminum slide, to cover a hole of 1 cm. diameter and 1.1 mm. depth. One drop of decalcified plasma
I. STUDIES ON THROMBOPOIESIS

was placed on the coverslip and the bone marrow tissue spread in it with a dissecting knife. Usually, one drop of Tyrode solution was added to the plasma, but this amount was varied according to the consistency of the clot required. The addition of 1 to 2 drops of chick embryo extract containing 1 mg 0.2M CaCl₂ was followed shortly by the clotting of the medium. The preparation was sealed with a second cover slip and kept in a 37°C incubator. During examination, the stage of the microscope was kept at the same temperature.

When large amounts of bone marrow not intended for direct observations, were cultured, the same proportions of ingredients were put into Carrel flasks but the medium was not recalcified and remained fluid.

The observations were carried out with a Wild phase microscope during 6-8 hours per day for up to six days. The megakaryocytes noted on the first day were repeatedly observed on the subsequent days and the changes occurring in them were described. Over 1000 megakaryocytes were thus observed in bone marrows of humans and experimental animals.

Thrombocyte Separation. Thrombocytes were separated from the bone marrow as follows. One ml. of bone marrow tissue was suspended in 5 ml. of Tyrode solution and centrifuged at 500 r.p.m. for five to seven minutes. The supernatant was removed and re-centrifuged at 3000 r.p.m. for fifteen minutes. This supernatant was discarded and the sediment (platelets) re-suspended in 0.2 ml. of Tyrode solution. The platelets were then counted and in 14 cases the serotonin* content was determined by biologic assays.9, 10

As an indication of the viability of thrombocytes produced in the bone marrow cultures, their ability to absorb serotonin was examined. It was important to establish whether dead thrombocytes absorb serotonin and the following experiment was devised. Equal numbers of platelets suspended in Tyrode solution were placed in six test tubes and to each of them 2.5 μg synthetic serotonin was added. The tubes were incubated at 37°C and one was removed each day, the thrombocytes washed, destroyed by freezing and thawing and the liberated serotonin determined in the supernatant fluid. As serotonin loses its biological activity within twenty-four hours at 37°C, the same procedure was repeated, serotonin being added only sixty minutes before the freezing and thawing.

The same procedure was employed with thrombocytes separated from bone marrow cultures. 1 μg serotonin† was added to the 0.2 ml. washed and counted platelet suspension, incubated for sixty minutes, washed three times, frozen and thawed and the supernatant was tested for serotonin. These determinations were performed at various intervals during 1-6 days of culture.

RESULTS

Direct Observations: The morphologic changes observed in the megakaryocytes of human and the various animals examined were similar and will be described together.

The young megakaryocyte in tissue culture is usually round or oval, with a clearly defined cytoplasm and a visible, slightly lobulated or round nucleus. The size of these cells varies from 25 μ to 100 μ. The cytoplasm is homogenous, finely granular, the granules being equally dispersed throughout the cytoplasm and showing intense Brownian movement (figs. 1a, b). The first visible changes occur in the cytoplasm 4-6 hours after explantation. The movement of the granules slows down and they tend to become coarser gradually filling the cytoplasm diffusely with round, fairly well defined, strongly refractile bodies (fig. 2). At the same time, the cellular membrane becomes blurred and irregular as if pieces had been torn out of the cell leaving irregular spaces (fig. 3). The nuclei undergo

* 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.

† Abbott Laboratories, North Chicago, Ill., U. S. A.
Fig. 1a.—Young megakaryocyte, showing clearly defined, finely granular cytoplasm and a slightly lobulated nucleus. (X 650.)

Fig. 1b.—The same as figure 1a at a higher magnification. (X 2000.)

Fig. 2.—Megakaryocyte containing coarse cytoplasmic granules dispersed equally throughout the cytoplasm. (X 2000.)

Fig. 3.—Megakaryocyte showing an irregular and slightly blurred outline. (X 2000.)

Fig. 4a.—Megakaryocyte with nucleus exhibiting coarse chromatin. (X 650.)

Fig. 4b.—Megakaryocyte in which differentiation between nucleus and cytoplasm is not possible. (X 1300.)

Fig. 5.—Megakaryocyte in which the coarse cytoplasmic granules have changed into elongated, strongly refractile irregular bands. (X 2000.)

Fig. 6.—Megakaryocyte showing breakdown into platelets and the spreading of newly formed platelets into the surrounding media. (X 2000.)
FIG. 7a, b.—Megakaryocyte with one portion transformed into platelets and the rest of the cytoplasm showing the changes illustrated in previous figures. (a, X 650; b, X 1300.)

FIG. 7c.—Note the large number of newly formed platelets surrounding the remnants of a megakaryocyte. (X 1300.)

FIG. 8.—Newly formed platelets as they appear 48–60 hours following the explantation of young megakaryocytes. (X 650.)

FIG. 9.—Elongated megakaryocyte containing coarse granulation. (X 2000.)

FIG. 10.—Megakaryocyte showing the beginning of pseudopodium formation. (X 2000.)

FIG. 11.—Megakaryocyte with irregular pseudopodia protruding in different directions. (X 2000.)
marked changes at the same time. The chromatin becomes coarser and the sharp limit between the nucleus and the cytoplasm gradually disappears (fig. 4a, b).

Twelve hours following the explantation various patterns of change can be followed.

The most frequent change encountered was the conglomeration of the cytoplasmic coarse granules, first into elongated, strongly refractile, irregular bands, leaving clear spaces between them (fig. 5). At this stage, the shape of the cells becomes irregular with small and large buds protruding. Between 24 and 48 hours the massive bands (columns) break up into smaller units (fig. 6), the distance between them steadily increasing (fig. 7a, b, c). These units separate into round and irregular elements with the characteristics of platelets as seen in the phase microscope. This procedure is usually completed within 48 to 60 hours (fig. 8), when the nucleus has already disintegrated.

Another pattern observed was the production of long pseudopodia at the coarse granular stage, one or two at a time, which underwent the changes described above (figs. 9–14). This pseudopodia formation continued until the megakaryocytes became transformed into platelets (fig. 15).

A rather rare form of platelet production observed was when the platelets formed actually in the cytoplasm of the megakaryocyte, without markedly altering the shape of the cell. When the procedure was completed, the platelets drifted away from the site of their production, leaving an empty frame, delineated by a strongly refractile homogeneous rim, which gradually disintegrated. These patterns occurred either separately but often combined with each other in the same cell.

In three instances mitosis of megakaryocytes was observed which was completed within twelve hours, following which, in one instance, the newly formed megakaryocytes broke down to thrombocytes as described.

In no instance could active mobility of megakaryocytes be observed; however, from time to time they seemed to have been moved passively by the surrounding mobile cells.

Polymorphonuclear leukocytes were frequently seen entering into the cytoplasm of the megakaryocytes (fig. 16). This could also be confirmed by the displacement caused by the leukocytes in the finer or coarser granules in the cytoplasm of the megakaryocytes. However in no instance was any change observed in the usual thrombocytopoietic process while the leukocytes were actually in the megakaryocytes or after they have left them.

In many instances red blood corpuscles were seen either attached to the surface of megakaryocytes or actually lying inside their cytoplasm (fig. 17). These intracellular red blood corpuscles were usually released after the breakdown of the megakaryocytes or if the megakaryocytes remained stationary, they gradually degenerated.

This process observed in the megakaryocytes continued for approximately 60 hours by which time most of the generating cells were entirely transformed into platelets. The nuclei following the completion of thrombocytopoiesis were usually either an unrecognizable mass of debris or round, shrunken and pyknotic.
I. STUDIES ON THROMBOPOIESIS

Fig. 12.—Two large pseudopodia showing break down into platelets. (X 2000.)

Fig. 13.—Newly formed platelets still contained within the original form of the pseudopodia. (X 2000.)

Fig. 14.—Megakaryocyte in which the upper and lower pseudopodia have broken down into platelets while the rest of the cytoplasm contains elongated irregular strongly refractile bands. (X 650.)

Fig. 15.—Newly formed platelets after the complete breakdown and dispersion of pseudopodia. (X 650.)

Fig. 16.—Large megakaryocyte showing several pseudopodia, in one of which the contours of five leukocytes can be distinguished. (X 650.)

Fig. 17.—Megakaryocyte containing several red blood corpuscles in the peripheral zone of the cytoplasm. (X 650.)

The changes described were seen in about 70 per cent of the megakaryocytes found in the culture. The remaining approximately 30 per cent of the cells, usually young and finely granular, remained stationary for the whole observation period. The number of cells without any morphologic evidence of platelet production varied with the individual man or animal. These cells did not show degenerative changes and there was no detectable morphologic difference between them and those which gradually broke down to give rise to a large number of thrombocytes.

On the fourth day of the experiment fibroblasts appeared in the culture multi-
plying steadily and 48 hours later the majority of the cells seen were fibroblasts over-growing the bone marrow tissue.

While this process continued, the other elements of the bone marrow also showed signs of activity. There was a marked migration of myeloid elements from the tissue fragments; phagocytosis was marked and in some instances red blood corpuscles were seen to undergo phagocytosis by white blood cells. Mi-

![Graph 1](image1)

**Fig. 18.**—Gradual decrease in the serotonin absorbing capacity of platelets incubated at 37 C. for 1-6 days in two separate series of experiments. This is due to the increasing number of degenerated platelets in the suspension.

![Graph 2](image2)

**Fig. 19.**—Thrombocytes separated from human bone marrow culture from 1-6 days. The number of thrombocytes increases but as no serotonin was added to the suspension, the newly formed platelets do not contain serotonin.
Fig. 20.—Similar curve to that illustrated in figure 19. 1 serotonin was added to the separated thrombocytes and accordingly their serotonin content increases proportionally to the increase in platelet counts.

toses in both myeloid and erythroid series were numerous. These observations indicate that the tissue culture was a living one and support the contention that the changes seen in the megakaryocytes are actually manifestations of their active function, rather than artifacts.

Quantitative and qualitative estimation of newly formed platelets: When equal numbers of washed, normal human platelets were incubated with serotonin the following results were obtained. There was a rapid decrease in the serotonin absorbing capacity of the platelets and in the 48 hours sample only traces of serotonin could be detected. The platelet count showed a slight but steady fall throughout the six days (fig. 18). As time progressed more and more platelets showed such degenerative changes as swelling, accumulation of the granules in one small area of the hyalomer and round empty shadows. As could be expected, the serotonin added 60 minutes before freezing and thawing in the second series of experiments could be regained from the supernatant fluid. These experiments indicated that non-viable platelets are not capable of absorbing serotonin.

The same procedure was repeated with thrombocytes isolated from cultures incubated from one to six days. The separated thrombocytes were re-suspended in each case in 0.2 ml. of Tyrode solution, counted and their serotonin content was determined. In each case there was a significant drop following the first 24 hours, both in the number of platelets and their serotonin absorbing capacity. However, after this period there was a gradual increase in the number of platelets and their serotonin absorbing capacity, which usually surpassed the values obtained before incubation (figs. 19–22).

These results indicate that the thrombocytopoiesis observed in vitro from megakaryocytes led to a significant increase in platelets which judged by their serotonin absorbing capacity were viable.
FIG. 21.—The same experiment as illustrated in figure 19. The bone marrow used was that of a guinea pig. No serotonin was added.

FIG. 22.—Guinea pig bone marrow culture. The addition of serotonin 60 minutes prior to the end of incubation causes an increase in the serotonin absorbing capacity of the thrombocytes which is proportionate to the rise in the platelet counts.

**DISCUSSION**

Many theories have been proposed as to the origin of the blood platelets. Thrombocytes have been said to originate from the plasma itself, from leukocytes, from red blood cells and others. All these theories are now of no more than historic interest. In 1906 Wright concluded that platelets are detached portions of the cytoplasm of megakaryocytes and since then much data have accumulated in support of his studies. It was found that platelets developed in the embryo at the same time as megakaryocytes. Furthermore an increased
The number of platelets in the peripheral blood is usually associated with a proportional increase of the megakaryocytes. Staining properties of platelet granules were found to be identical with those of the megakaryocytes, and "anti-bone marrow sera" were found to remove the platelets from the circulation. The fine structure of the megakaryocyte cytoplasm was found to be similar to that of the thrombocytes and early forms of thrombocytes were observed under the electron microscope within the cytoplasm of the megakaryocytes. In pathologic conditions within which the platelets were affected, as in idiopathic thrombocytopenic purpura, both quantitative and qualitative changes were observed in human megakaryocytes.

All these observations were based mainly on morphologic evidence and the quantitative relationship between platelets and megakaryocytes, and were thus indirect and inconclusive. Direct observation on actual production of platelets was reported by Bedson and Johnston in 1925, who followed the break-down process of human megakaryocytes in bone-marrow smears preserved on a slide for three days. They concluded that as the bulk of the cells perished, the break-down of the megakaryocytes must be considered as an artifact. Another more recent reference was made to similar observations by Japanese workers, but details of their work were not available. In a recent study Theyry and Bessis observed platelet production in surviving bone-marrow tissue from mice and rats which was photographed continuously through the phase microscope. Their observations lasted up to twelve hours, and the morphologic changes described are similar to those found during the course of the present study.

In the present study the production of blood platelets from megakaryocytes in tissue cultures was studied by continuous direct observation. This method seemed most appropriate as it enabled the observation of the same cell at various intervals up to six days.

The changes observed in the megakaryocytes of healthy human beings and various experimental animals were similar.

In all cases the finely granular structure of the cytoplasm of the young, usually round megakaryocyte was gradually replaced by coarse granules, which then fused to give rise to irregular, strongly refractile bands. The cell became oval or irregular with pseudopodia, filled with the bands already mentioned. During the next stage the bands broke down into platelets filling the whole microscopic field. The nuclei usually disintegrated, or when present, were shrunken and pyknotic. This process was usually completed by the end of 60 hours following the explantation of the bone marrow. It is of interest that a number of apparently young undamaged megakaryocytes remained unchanged for the whole period of observation. No explanation could be found for the lack of activity of these cells on the basis of their morphologic properties as seen in the phase microscope.

It has been suggested that the megakaryocytes are broken down to platelets by the polymorphonuclear leukocytes, which enter into the megakaryocytes and disrupt their cytoplasm mechanically. This suggestion can be ruled out from our own observations. Leukocytes were seen repeatedly to "cross" megakaryocytes without any noticeable effect on the usual process of thrombopoiesis.

No definite conclusion could be drawn with regard to the phagocytic ability of the megakaryocytes. In several instances erythrocytes were seen in the cyto-
plasm. Whether they were actively incorporated by the megakaryocyte or were at first attached to it and then entered the cell when its membrane disintegrated, remains a problem to be elucidated.

The semi-solid consistency of the culture medium was highly favorable for ameboid movements as seen by the vigorous and relatively rapid motion displayed by the leukocytes and reticulum cells. In spite of this in no instance was there a change in the location of a megakaryocyte, when its surrounding was free of cells capable of active movement. The displacement of megakaryocytes by groups of leukocytes was frequently seen.

It has been established that the carrier of serotonin in the peripheral blood is the platelet, while the other cellular elements of the peripheral blood, except possibly for the mast cells of the bone marrow, do not contain serotonin. The application of this observation in our experiments seemed to provide a method for labeling the platelets formed and at the same time to serve as an indicator of their functional capacity and viability. Our preliminary investigations have shown that thrombocytes degenerating during incubation at 37 C. for two to six days are not capable of absorbing added serotonin. Furthermore it was found that the serotonin absorption capacity of a platelet suspension is proportional to the number of living platelets in the suspension. Thus the "serotonin absorbing capacity" of platelets was introduced as an indicator of the viability of the newly formed thrombocytes as well as an indirect quantitative measure of their production. With this method it was found that an increasing number of viable platelets were produced during the five to six days of incubation under the experimental conditions described.

It should be borne in mind that the observations on thrombocytopoiesis reported were made in tissue cultures under artificial conditions which deprived the explanted tissue of its continuous contact with the rest of the living organism. Thus the assumption that the thrombocytopoiesis observed in vitro reflects what actually happens in the bone marrow may be premature. However the method employed here provided a useful tool for the examination of normal bone marrow as well as those from patients with idiopathic thrombocytopenic purpura and the effect of anti-platelet serum on platelet production. The results of these investigations will be published in a separate paper.

SUMMARY

Thrombocyte production from megakaryocytes of healthy humans, dogs, guinea pigs and mice was observed continuously for one to six days in tissue culture.

Approximately 70 per cent of the explanted megakaryocytes broke down to give rise to numerous platelets, while the remaining 30 per cent of the cells remained unchanged.

The newly formed thrombocytes were separated from the rest of the bone marrow tissue, counted and their serotonin absorbing capacity determined.

There was invariably a gradual increase in both the number of thrombocytes and in their serotonin absorbing capacity during the one to six days of observation.
I. STUDIES ON THROMBOPOIESIS

The results obtained were similar in human megakaryocytes and in those of experimental animals.

SUMMARIAL IN INTERLINGUA

Le production de thrombocytos ab le megacaryocytos de humanos imi bon sanitate, de canes, porcos de India, e muses esseva observate continuamente in histoculturis durante periodos de inter 1 e 6 dies.

Approximativemente 70 pro cento del megacaryocytos explantate se disintegra, facente nascer numerose plachettas, durante que he remanente 30 pro cento del megacaryocytos remaneva intacte.

Le novensente formate thrombocytos esseva separate ab le resto del medulla ossee, illos esseva contate, e lor capacitate de absorber serotonina esseva determinate.

Sin exceptiomi il habeva un augmento gradual in le numero de thrombocytos e etiam in lor capacitate de absorber serotonina durante le periodo de observation.

Le resultatos obtenite esseva simile in le studios de megacaryocytos human e animal.

REFERENCES

G. IZAK, D. NELKEN AND J. GUREVITCH

Studies on Thrombopoiesis: I. Thrombocytopoiesis in Vitro: Experiments with Animal and Normal Human Material

G. IZAK, D. NELKEN, J. GUREVITCH and MISS A. HERZOG