A Study of Thrombopoiesis in Induced Acute Thrombocytopenia

By Martin Matter, John R. Hartmann, Jean Kautz, Quin B. DeMarsh and Clement A. Finch

There is at present only limited information concerning thrombopoiesis in animals and man. While the normal turnover of platelets has been generally established through life span measurements employing suitable platelet tags, the response in platelet production to an increased need for platelets has not been well defined. Observations on replenishment of circulating platelets following their depletion through exchange transfusion have been reported in the dog by Craddock and associates and in man by Krevans and Jackson. Yamada has shown that information concerning platelet formation may be obtained by the examination of the fine structure of the megakaryocyte employing the electron microscope. In this report these technics have been combined to indicate the nature of the changes which occur in both megakaryocytes and circulating platelets as a result of induced thrombocytopenia. Further studies are included to indicate the alteration of the thrombopoietic response which occurs as a result of abnormalities in splenic function.

Methods

Sprague-Dawley rats of either sex weighing between 250 to 375 Gm. were used. Studies were done on normal rats, on rats after splenectomy, and on rats after 15 weeks of methylcellulose injections. Platelet depletion was produced by bleeding the animals from the femoral artery and replacing this blood with donor blood which had been depleted of platelets.

Platelet-poor blood was prepared from rat blood removed by cardiac puncture into siliconized syringes through monocoted 20 gage needles. Sodium sequestrene (1 mg. per ml. of blood) was used as an anticoagulant. Platelet-free plasma was prepared by an initial centrifugation of blood at 3000 rpm (1500 g) for 15 minutes and by subsequent high-speed centrifugation of the separated plasma. The red cells were resuspended in 0.9 per cent saline and centrifuged at 1000 rpm (180 g) for 15 minutes six to eight times. Each time the supernate containing the platelets was removed and discarded. At the completion of these separations, the platelet-free plasma was mixed with the packed cells. By this procedure 40 to 60 ml. of blood which contained 14 to 16 Gm. of hemoglobin per 100 ml., 6000 to 8000 leukocytes per cu.mm. and only 4000 to 12,000 platelets per cu.mm. were prepared for each exchange transfusion. The blood was warmed to 38 C. shortly before its administration.

The exchange transfusion* was performed in animals anesthetized by the intraperitoneal...
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injection of Nembutal (4 mg. per 100 Gm. body weight). A PE-50 polyethylene catheter was introduced into the femoral vein and a siliconized 20 ml. syringe containing platelet-poor blood was affixed by means of a monocoted 23 gage needle to this catheter. A second catheter was placed in the femoral artery on the same side. Two milliliters of blood were immediately collected by normal arterial flow into a siliconized tube and replaced with 2 ml. of platelet-poor blood injected slowly through the venous catheter. After a mixing time of approximately one minute had elapsed, 2 ml. of blood were again removed from the artery and replaced by platelet-poor blood through the vein. By repeating this procedure, a total of 30 to 60 ml. of blood was exchanged over a one hour period. The catheters were then removed, the vessels ligated, and the skin closed. To counteract the chemical action of sequestrene, a solution containing 1.5 Gm. of calcium chloride and 1 Gm. of magnesium chloride in 100 ml. of saline was injected intravenously during the transfusion in amounts of 0.25 ml. for each 10 ml. of platelet-free blood.

Platelet counts were performed in duplicate before and at intervals after the exchange transfusion, using blood obtained from a free-flowing cut in a tail vein. Blood samples for platelet counting were taken at approximately the same time each day (10:00 a.m.), and all counts were done by the same observer. Platelets were diluted in Feissley’s solution and a phase microscope was employed for the counting procedure. The coefficient of variation determined from 22 sets of duplicate counts was 8.0 per cent. The normal rat platelet count based on 38 determinations was found to be 840,000 per cu.mm. (SD ± 175,000).

Megakaryocyte counts were performed on the femoral marrow of animals sacrificed at intervals during the experiment. The femur from the intact leg was removed, and the cylinder of marrow was carefully removed in one piece. The marrow was fixed in Bouin’s solution, imbedded in Tissuemut and cut longitudinally in 10 micron sections. Sections were stained with hematoxylin and counter-stained with azophloxine. Megakaryocytes in an entire section of marrow were counted and an average was taken from counts of about 12 sections. The total area of a marrow section was determined by planimetry of an enlarged photograph of the section. Megakaryocytes per sq.mm. of marrow were then calculated from the number of megakaryocytes in the section divided by the area of the section in sq.mm.

Marrow was prepared for electron microscopy in the following manner. The femur or humerus from the intact side was removed and a small amount of fixative injected directly into the marrow chamber. The marrow was removed and cut into pieces of less than 1/2 cu.mm., fixed at 0 C. for two hours in 0.2 per cent osmium tetroxide buffered with veronal acetate to pH 7.2 to 7.4, and potassium chloride was added to achieve isotonicity. Dehydration in ethanol was carried out rapidly and without intermediate washing after fixation. Tissue was imbedded in a 9:1 solution of N. butyl: methyl methacrylate. Polymerization was carried out at 40 C. with the aid of Luperco CDB as catalyst. Sections in which the thickness was estimated to be 1/20 or 1/40 micron (displaying gold or silver interference colors) were cut on an ultramicrotome. The sections were mounted on carbon-coated Formvar films on 150 mesh Lektromesh specimen grids. They were viewed in an RCA-EMU electron microscope equipped with a 10 micron objective aperture. Electron micrographs of appropriate areas were taken at relatively low magnifications (3000 to 6000 times) on Kodak Medium lantern slide plates. Magnification was calibrated with Dow polystyrene latex particles.

Platelet concentrates were prepared for electron microscopy in the following manner. Blood was drawn from the abdominal aorta of anesthetized rats into cooled, siliconized syringes containing sequestrene in saline. The blood was transferred to a siliconized centrifuge tube and spun for 20 minutes at 800 rpm (110 g) at 4 C. to remove red and white cells. Platelets were then centrifuged from the plasma at 1500 rpm (375 g) for 20 minutes. The button of platelets was then resuspended in buffered osmic acid fixative. Fixation, dehydration and embedding were carried out in a similar fashion to that described for marrow.

RESULTS

The depletion of circulating platelets.—In four rats an exchange transfusion was carried out with fresh rat blood drawn into siliconized equip-
Theoretical loss

0 20 160 200%

blood volume exchanged

Fig. 1.—Exponential loss of platelets removed by exchange transfusion with platelet-poor blood. The depletion of circulating platelets as a function of volume of blood exchanged is shown. The four animals studied are indicated by different symbols. Each dot represents an individual platelet count. The line drawn is the theoretical depletion based on a calculated blood volume and the amount exchanged with the assumption that no viable platelets were transfused.

The thrombopoietic response to thrombocytopenia.—The data of platelet response in six animals whose platelet counts were reduced to approximately 10 per cent of normal are shown in figure 2. There was a gradual rise over the first two days after exchange transfusion, a more rapid increase during days three and four reaching supranormal levels, and a decrease to normal by the seventh to ninth day. Megakaryocyte counts over this time interval showed a slight increase immediately after the exchange, but an increase of 27 per cent above normal during the time when platelets appeared in the blood at an increased rate.

Electron microscope studies of megakaryocytes and circulating platelets were correlated with these quantitative changes. The normal megakaryocytes of the rat marrows typically showed large polymorphic nuclei with several nucleoli and chromatin which was often clumped. Mitochondria, Golgi membranes, endoplasmic reticulum and numerous small, dark granules were present in the extensive cytoplasm. The most unique feature was the paired demarcation membranes within the outer zone of endoplasm (fig. 3A), as has also been described by Yamada. The enclosed areas and their contents...
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Fig. 2.—Changes in platelet and megakaryocyte counts following acute platelet depletion. The effect of platelet depletion on numbers of thrombocytes and megakaryocytes is shown. The shaded area in the platelet curve represents one standard deviation of serial platelet counts in six animals. The megakaryocyte counts are based on observations in individual animals at the times indicated.

resembled platelets seen in the peripheral blood. However, we were not able to observe platelets in the process of separation from megakaryocyte cytoplasm.

Striking changes in megakaryocytes were observed by electron microscopy immediately after the exchange transfusion. There was a reduction in cytoplasmic mass, and the platelet demarcation membranes previously seen in the outer cytoplasm were no longer present (fig. 3B). These changes were maximal on the day of the exchange transfusion, but were still evident three days later. Platelet demarcation lines had returned by the seventh to fourteenth day.

Ocular microscopic measurements were made on the diameter of 30 megakaryocytes in histologic sections before and at intervals after exchange transfusion. The megakaryocytes showed an average diameter of 21.1 μ ± 4.6 before as compared to 17.9 μ ± 2.6 afterwards. At 24 hours an average diameter was 17.6 μ ± 3.7, and by the third day the average had risen to 20.5 μ ± 2.7. These changes on day zero and day one are considered significant (P less than 0.01).

There was no change as visualized by electron microscopy in the appearance of platelets obtained immediately after the exchange transfusion nor at 24 or 48 hours, and no large cytoplasmic fragments of megakaryocytes were observed in platelet concentrates from the circulating blood at these time...
Fig. 3A.—Megakaryocytes from normal marrow. Most of the megakaryocytes show a polymorphic nucleus and extensive cytoplasm divided into numerous platelet units. Platelet demarcation membranes outline these units, each of which contains some cytoplasm (hyalomere), an occasional vacuole and small dark round specific granules. A few larger ovoid mitochondria are seen, especially near the nucleus.

intervals. However, on the third and fourth days platelets of larger than normal size with decreased granulation were observed (fig. 4A and 4B).

*Effect of repeated exchange transfusion on thrombopoiesis.*—To determine whether thrombopoiesis could be further increased by a stimulus of greater magnitude, one animal was subjected to exchange transfusion with platelet-poor blood on two successive occasions. As shown in figure 5, the second response was more rapid in its occurrence and of greater magnitude than that observed after a single period of thrombocytopenia.

*Effect of the spleen on platelet production.*—To provide further information on the interrelation between megakaryocytes and circulatory platelets, animals were studied in which platelet counts averaged 16 per cent above normal as a result of splenectomy or were reduced to 36 per cent of normal by injection of methylcellulose. Megakaryocyte counts of animals in each group showed little deviation from the normal either initially or following platelet depletion.
Fig. 3B.—Megakaryocytes one day after exchange transfusion. Platelet units are no longer seen. Vacuoles are larger and more numerous. Specific granules are less numerous than in normal megakaryocytes. The close proximity of a megakaryocyte to a capillary and the presence of numerous "vesicles of Palade" along the endothelial cell membranes are suggestive of active transport of substances across the endothelium. Similar vesicles are seen in the capillaries of many other active tissues (endocrine, lung, kidney).

The time relationship of response to induced thrombocytopenia was similar to normal in both groups of animals, but the amplitude was proportional to the basal platelet count (fig. 6). Thus in the splenectomized animals thrombocytosis on days 3 and 4 was normal or above, whereas the response of the methylcellulose animals was approximated one-third of normal. To exclude the possibility that methylcellulose might have impaired platelet response through marrow damage or through effects secondary to renal disease, splenectomy was performed on these animals. Figure 7 shows their unimpaired platelet response to splenectomy.

Discussion

Studies by Craddock et al. in dogs depleted of platelets by blood exchange indicated no platelet reserve, and similar data have been obtained
Fig. 4A (at top).—Platelets, one hour after exchange transfusion. The remaining platelets appear normal. Six to 12 dark rounded granules are scattered throughout the hyalomere of each sectioned platelet.

Fig. 4B (at bottom).—Platelets, three days. The platelet count is approaching its highest point. These platelets are larger and less granular than normal. This difference in cross section becomes considerably greater when expressed as volume change.

by us. There was no evidence at the termination of the depletion procedure that any circulating platelets had been mobilized to replace those removed (fig. 1). In addition, there was no evidence of alteration in the rate of platelet production during the 48 hours following exchange transfusion (fig. 8). The two day delay in thrombopoietic response is somewhat shorter than the three to four days observed in the dog and approximately five days observed in man. However, the lag phase is perplexing when the immediate loss in cytoplasmic mass by the megakaryocytes and disappearance of their platelet demarcation membranes is considered. One might speculate that
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Fig. 5.—Platelet response in an animal subjected to two exchange transfusions of 42 ml. each. The second recovery period was shorter and the response somewhat greater than that observed in singly depleted animals.

Fig. 6.—The normal platelet regeneration curve is indicated within the shaded area. Regeneration curves of splenectomized animals are indicated by dotted lines and of animals with splenomegaly induced by methylcellulose injections, by solid lines.
Fig. 7.—Studies of platelet counts after splenectomy in two normal and two methocellose-injected rats are illustrated. It will be observed that thrombocytosis as a result of splenectomy is unimpaired in the methycellulose-treated animals.

Fig. 8.—Platelet production following acute platelet depletion. The normal platelet regeneration curve (fig. 2) has been recalculated, with the assumption of a normal production of 20 per cent per day. Since approximately 90 per cent of existing platelets are removed and new platelets may all be expected to live for five days, daily destruction of platelets is considered negligible. The increase in platelet count then reflects production. It is evident that increase in thrombopoiesis is limited to the third and perhaps fourth day of the study.

these cytoplasmic fragments were temporarily occupying an intravascular but noncirculating position in some such organ as the lungs. However, since the shedding of cytoplasmic fragments by megakaryocytes was not directly observed, and since no fragments were found in circulation, the interpretation of these findings must remain an open question.

On the third day following platelet depletion there was an abrupt increase in thrombopoiesis to approximately four times normal (fig. 8). This occurred with only a 25 per cent increase in the number of megakaryocytes. It is of interest to compare this thrombopoietic response with that occurring in the erythron, since both systems have in common a marrow precursor population and an adult cell population contained within the circulating blood. Under the stimulus of severe anemia there may be a rapid shift of the marrow reticulocyte pool to the blood, thereby doubling the entry of red cells into circulation in any one day; however, any subsequent increase in erythropoiesis is dependent on a proportionate increase in nucleated cell population of the
marrow. In the present studies a proportionate increase in precursor cells was not observed, indicating that the platelet response to acute thrombocytopenia may be largely achieved through a cytoplasmic response of megakaryocytes.

The association of splenic pathology and alteration in concentration of circulating platelets is well documented in clinical reports. In the present experimental study similar changes were observed, in that splenectomy resulted in thrombocytosis and splenomegaly was associated with thrombocytopenia. Studies of platelet survival in these two conditions reported elsewhere indicate that there is no significant alteration in platelet life span in either splenectomized or methylcellulose injected rats, and that the splenic effect therefore involves platelet production rather than destruction. Further evidence for this thesis is provided by megakaryocyte counts of the marrow of these animals; the observed changes in megakaryocyte counts were in the same direction as the alteration in platelet counts (table 1). Thus, splenectomized animals show a 23 per cent increase in megakaryocytes and a 42 per cent increase in platelets, while methylcellulose animals show a 13 per cent decrease in megakaryocytes and a 61 per cent decrease in circulating platelets. In all groups of animals the induced acute thrombocytopenia resulted in increases in megakaryocytes on the third day of 27 per cent in the normal group, 24 per cent in splenectomized animals and 57 per cent in methylcellulose-treated animals. The increase in platelet production was of far greater magnitude than the increase in megakaryocytes. The increase in circulating platelets following depletion was generally proportional to the initial platelet level, implying that the splenic effect was maintained through the period of thrombopoietic stimulation.

As a final point in the discussion it should be made clear that the observations reported here reflect only the sequence of events after a very temporary period of thrombopenia. Repeated thrombopoiesis leads to a further increase in platelet production beyond the response measured in these studies. While platelet response in these studies occurred largely through cytoplasmic changes, multiplication of megakaryocytes might be expected to be of more importance with a more prolonged thrombocytopenic stimulus.

Conclusions

Changes in circulating platelets and marrow megakaryocytes in the normal rat after acute platelet depletion have been examined. A decrease of circulat-

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ing platelets to 10 per cent of normal levels was produced by exchange transfusion. This depletion occurred in proportion to the amount of platelet-free blood exchanged, and no evidence of reserve stores of platelets was observed.

Immediately after the induction of thrombocytopenia, there were marked changes in the morphology of marrow megakaryocytes, consisting of a decrease in cytoplasmic mass and the loss of cytoplasmic demarcation membranes.

There was a latent period of two days before the rate of appearance of platelets in the circulating blood was increased. After a temporary thrombocytosis normal levels of platelets were established by the seventh day. Newly appearing platelets were larger than normal. Marrow megakaryocytes increased slightly at the time of increased thrombopoiesis, but not in proportion to the increased rate of platelet production.

In studies of thrombocytosis induced by splenectomy, marrow megakaryocytes were slightly increased. When splenomegaly was induced by methylcellulose, the number of megakaryocytes was slightly decreased. The evidence presented here, and platelet life span measurement in similarly prepared animals reported elsewhere, indicate that the spleen exerts its effect on the level of circulating platelets through alteration in the rate of thrombopoiesis.

In all animals it appeared that the response to acute thrombocytopenia involved predominantly an alteration in cytoplasmic production, rather than an increase in the number of megakaryocytes.

**Summario in Interlingua**

Esseva examinate in rattos normal le alterationes occurrente in le plachettas circulante e in le megacaryocytos medullar post depletion acute del plachettas. Un reduction del numeration plachetta in le circulation usque a un nivello amontante a 10 pro cento del valores normal esseva effectuate per transfusion de excambio. Le depletion del plachettas in le circulation occurreva in proportion directe con le quantitate de displachettate sanguine usate in le transfusion de excambio, e nulle indicio pro le presentia de reservas de plachettas esseva observate.

Immediatemente post le induction de thrombocytopenia, marcate alterationes deveniva manifeste in le morphologia del megacaryocytos medullar. Iste alterationes consisteva de un reduction del massa cytoplasmic e del perdita del membranas de demarcation in le cytoplasma.

Esseva notate un periodo latente de duo dies ante que le apparition de plachettas in le sanguine del circulation ganiava in intensitate. Post un transiente thrombocytosis, nivello normal esseva establite pro le plachettas le septime die post le transfusion de excambio. Le nove plachettas esseva plus grande que plachettas normal. Le numeration del megacaryocytos medullar se augmentava levemente al tempore del augmentate thrombopoiese, sed non in proportion al augmento de intensitate in le production de plachettas.

In studios de thrombocytosis inducite per splenectomia, le numeration del megacaryocytos medullar esseva levemente augmentate. Quando spleno-
meagalia esseva inducite per methylcellulosa, le numero del megacaryocytos esseva levemente reducite. Iste observationes, insimul con mesureaciones del superviventia plachettal in similemente preparate animales (que esseva reportate alterubi), indica que le splen exerce su efecto super le nivello del plachettas in le circulation per alterar le intensitate del thrombopoiese.

In omne le animales il pareva que le responsa a thrombocytopenia acute consisteva predominantemente do un alteration del production cytoplasmic plus tosto quo do un augmento del numoro do megacaryocytos.

REFERENCES


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