Megakaryocyte Maturation Rate in Thrombocytopenic Rats

By Shirley Ebbé, Frederick Stohlman, Jr., Janet Donovan and Joan Overcash

Three stages of maturation of rat megakaryocytes are recognizable morphologically, and their total maturation time has been estimated to be about 2-3 days. Maintenance of megakaryocytopoiesis has been shown to be dependent on continuous influx from unrecognizable precursor pools rather than on proliferation within the compartment of recognizable megakaryocytes.

In response to acute thrombocytopenia, megakaryocytes of experimental animals may increase in number and an increase in the proportion of immature forms has been described. For changes in megakaryocyte number or differential to occur, there must have been an imbalance between differentiation of precursor cells into megakaryoblasts and disintegration of granular megakaryocytes into platelets. This might be due to changes in rate of differentiation and/or changes in maturation rate of recognizable megakaryocytes. In the present report, megakaryocytopoiesis was observed in rats during the phase of recovery from acute thrombocytopenia to study any such compensatory changes which might occur.

Materials and Methods

Male Sprague-Dawley rats which received heterologous antiplatelet serum weighed 170-240 Gm., and those which were exchanged transfused or sham operated weighed 260-360 Gm. Larger rats of the same strain were used as blood donors. Platelet counts were done by phase microscopy on cardiac blood anticoagulated with dry K₂EDTA. Each test animal was used for only one determination; to accumulate a series of determinations, groups of rats were treated and sequentially sacrificed.

Bone marrow smears were prepared by a paint brush technic from a split femur. After exchange transfusion or operation, the femur from the intact leg was used. The smears were stained with Wright's and Giesma stains for differential counts of megakaryocytes which were classified as previously described into stages I, II, and III corresponding to megakaryoblast, promegakaryocyte, and granular megakaryocyte, respectively. For each rat, 500 megakaryocytes were classified. Labeling of megakaryocytic nuclei was accomplished by injection of tritiated thymidine (³HtdR) intravenously at a dose of 1 micro-

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curie per Gm. body weight (Schwarz BioResearch, Inc., 1.9 curies/mmole, 1 millicurie/ml.) Autoradiographs were prepared and evaluated as previously described. For each rat, nuclear grains over 250 megakaryocytes were counted.

To immunize rabbits to rat platelets, 3 intravenous injections of rat platelets suspended in saline were given at 3 and 5 day intervals. Each injection contained 10–19 × 10^9 rat platelets. Serum was collected 2 weeks after the last injection and was heated at 56 C. for 1 hour then absorbed 3 times with equal volumes of rat erythrocytes separated from defibrinated blood and washed 3 times. The serum was then frozen until use. To produce thrombocytopenia, each test rat received 0.2 ml. of this serum intraperitoneally.

Exchange transfusion with platelet poor homologous blood was done by the technique of Matter et al. as previously described. Donor blood was collected into ACD solution by cardiac puncture of rats anesthetized with ether. Platelets were removed from the red cells by repeated centrifugation (150–225 g.) and resuspension in saline. The red cells were finally resuspended in platelet poor plasma. The final hematocrits were 40–50 per cent, and platelet counts were less than 10,000/mm³. The blood was stored at 4 C., used within 48 hours of preparation, and warmed in an incubator at 37 C. before use.

Rats for exchange transfusion were anesthetized with chloral hydrate or nembutal, and the femoral artery and vein on one side were cannulated. Exchange transfusion was accomplished in 2 ml. increments, utilizing 50 ml. of platelet poor blood. After each 10 ml., calcium, equivalent to 10 mgm. calcium gluconate monohydrate, was injected, I.V. Tetracycline HCl powder was dusted into the wound after removal of catheters and vessel ligation and before suturing and closing with skin clips.

Sham operated rats were subjected to anesthesia and surgery as in exchange transfused rats except that the femoral artery and vein were simply ligated.

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**Table:**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Days</th>
<th>Average ± S.E.</th>
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<tbody>
<tr>
<td>Stage I</td>
<td>1 2 3 4 5 6 7 8</td>
<td>Treated</td>
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<tr>
<td>Stage II</td>
<td></td>
<td>Controls</td>
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<tr>
<td>Stage III</td>
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**Fig. 1.—Differential counts of megakaryocytes after decrease in platelet levels to ~8 per cent of normal by exchange transfusion. On and after 1 day, each point represents the average for 5–11 rats; the 30 min. and 8 hr. points are averages for 3 rats.**
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Results

Platelet Counts

Platelet counts immediately after exchange transfusion were \( \sim 8 \) per cent of the pre-transfusion values (average value for 97 exchanged rats was \( 0.0856 \times 10^6/\text{mm}^3 \) vs. control values of \( 1.127 \times 10^6/\text{mm}^3 \)). For the first two days thereafter, average platelet counts increased by increments of \( \sim 300,000/\text{mm}^3/\text{day} \). During the third post-thrombocytopenic day the average platelet count increased by \( \sim 600,000/\text{mm}^3 \).

After injection of antiplatelet serum, platelet counts dropped to <0.1 per cent of the control values (average of \( 4 = 1,000/\text{mm}^3 \) vs. \( 1.143 \times 10^6/\text{mm}^3 \), the average of 23 controls). Platelet counts remained low one day later (average of \( 5 = 32,500/\text{mm}^3 \)), but gradually increased to supranormal values thereafter, with increments of \( \sim 400,000/\text{mm}^3 \) and \( \sim 700,000/\text{mm}^3 \) occurring during the third and fourth days, respectively.

Megakaryocyte Differentials

Differential counts of megakaryocytes of the three stages of maturation were done after reduction of the platelet count to \( \sim 8 \) per cent of normal by exchange transfusion. In Figure 1 the results for eight days after acute
thrombocytopenia are presented. Values in treated animals were comparable to those in the controls. Differential counts were done at intervals for three days after development of more severe thrombocytopenia from antiplatelet serum and after sham operation. The numbers of rats were small, with only 2–5 animals observed at each interval, but there was no immediate or delayed change apparent in megakaryocyte differentials after either procedure.

**Megakaryocyte Labeling with $^3$HTdR**

$^3$HTdR was used to estimate rates of maturation of recognizable megakaryocytes, and labeling was measured autoradiographically for two days after its injection. To determine if there was an early direct effect on differentiated megakaryocytes by platelet depletion, $^3$HTdR was injected either 30–60 minutes before initiation of exchange transfusion with platelet poor blood or one hour after injection of rabbit anti-rat platelet serum. For the first 24 hours, labeling indices in thrombocytopenic rats were similar to their controls (Fig. 2). Two days after injection of $^3$HTdR, 98–100 per cent of each stage of megakaryocytes in the thrombocytopenic rats were labeled. In normal rats, this degree of labeling in stages I and II at this time was not uncommon, but stage III had never become this completely labeled before the third
day. This finding suggested a delayed effect of thrombocytopenia on megakaryocytopoiesis with acceleration of maturation between 24 and 48 hours after depletion.

Accordingly, labeling of megakaryocytes was observed when \(^{3}\)HTdR was injected 24 hours after platelet depletion by exchange transfusion or anti-platelet serum (Fig. 3). Thirty minutes after injection of \(^{3}\)HTdR, only stage I megakaryocytes were labeled, as usual, but a somewhat higher proportion of these were labeled in thrombocytopenic rats (34–45 per cent) as compared to controls (18–28 per cent). Thereafter, the label increased in stage I and appeared in stages II and III in the same sequence as normal. However, maturation through the three stages was more rapid than normal as indicated by higher labeling indices in all stages for the first 24 hours.

Grain counts in stage II at eight hours and in stage III at one day were of the same order of magnitude as in stage I at 30 minutes indicating that, as in normal rats, the label was not diluted by cellular division of megakaryocytes. Grain counts over labeled cells in thrombocytopenic rats showed no consistent differences from those of the controls.

During the three days after sham operation, the animals did not develop thrombocytosis. Nevertheless, megakaryocyte maturation was accelerated between one and three days postoperatively (Fig. 4). In previous observations, thrombocytosis did not develop until the fourth post-operative day.

**DISCUSSION**

In normal rats of the strain used in these experiments, the destruction of ~40 per cent of circulating platelets daily, as measured with an in vivo triti-
ated diisopropylfluorophosphate label, indicated daily production of \( \sim 440,000 \) platelets/mm\(^3\) to maintain the normal platelet count of \( \sim 1.1 \times 10^6/mm^3 \). During regeneration of circulating platelets after acute platelet depletion by exchange transfusion this daily increment was not exceeded until the third post-thrombocytopenic day. This is in agreement with the observations of others that there is a lag period of about two days before the rat accelerates platelet production in response to thrombocytopenia and correlates with acceleration of maturation of megakaryocytes seen in the present studies during the second post-thrombocytopenic day. In the animals treated with antplatelet serum, the onset of recovery was delayed by one day. Normal daily platelet production was not exceeded until the fourth day in spite of accelerated maturation of megakaryocytes during the second day. The reason for this delay was not clear.

Megakaryocytes have been shown to reutilize tritium, thus making long-term labeling patterns difficult to interpret, but for the first day or two after "flash" labeling, the labeling indices give a reliable estimate of maturation time of recognizable megakaryocytes. Little information can be derived from absolute grain counts in small numbers of animals because of reutilization and the wide range of grain counts seen in groups of normal rats.

In thrombocytopenic rats, as in normal rats, only stage I megakaryocytes labeled initially with \(^3\)HTdR, and appearance of label in stages II and III was dependent on maturation. When labeling was accomplished one day after the thrombocytopenic stimulus, the rapid increase of labeling index in stage I and rapid rate of appearance of label in stages II and III in the presence of normal differential counts indicated both an increased rate of influx into the compartment and an increased maturation rate of megakaryocytes formed during thrombocytopenia. When \(^3\)HTdR was given within an hour of production of thrombocytopenia, these parameters were normal for the first 24 hours. However after 48 hours, stage III megakaryocytes were nearly 100 per cent labeled. Thus, the pre-existing, nonlabeled stage III megakaryocytes had been almost completely replaced by the end of the second day after platelet depletion. Since labeling of all stages was normal at the end of the first day, this finding suggested that, between 24 and 48 hours after production of thrombocytopenia, the maturation of all megakaryocytes was accelerated—those pre-existing as well as those formed in response to thrombocytopenia.

Assuming that all cells which entered the megakaryocyte compartment after injection of \(^3\)HTdR would be labeled, total transit time \( T_{mk} \) could be estimated from the rate of replacement of unlabeled megakaryocytes of all stages by labeled cells. This assumption is given credence by the prompt development of nearly 100 per cent labeling in stage I megakaryocytes seen repeatedly in normal rats. By this method, \( T_{mk} \) of normal rat megakaryocytes was previously estimated to be about 60 hours. In the present experiments, the pooled control data for the first 24 hours after injection of \(^3\)HTdR showed a \( T_{mk} \) of 72 hours which is within the range for previous estimates for total maturation time of 43–75 hours. During the first day after platelet depletion by exchange transfusion or antiserum, values were 75 and 70 hours; during
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the second day, they were 43 and 32 hours, respectively, or 44–60 per cent of control. In sham operated rats, \( T_{mk} \) was reduced to 51 hours (71 per cent of control) during the second postoperative day. Thus, the procedure itself was responsible for some of the shortening of megakaryocyte maturation time seen in rats made thrombocytopenic by exchange transfusion. That platelet depletion also influenced maturation is indicated by the more rapid maturation in the thrombocytopenic rats and by the prominent acceleration of maturation of megakaryocytes in recipients of antiplatelet serum.

Substantial drops in platelet counts have been described during\(^{13}\) or after\(^{14}\) major surgery in human beings which were out of proportion to the amount of hemorrhage and transfusion with banked blood. It is possible that the sham operated rats developed thrombocytopenia during surgery which stimulated megakaryocyte maturation, but their platelet counts were normal one day after the procedure. Also, the labeling pattern after sham operation (Fig. 4) was different from that seen when thrombocytopenia was superimposed (Fig. 3). Labeling indices of all three stages increased more rapidly than normal during the second post-thrombocytopenic day, but only stages I and II were affected during the same period after sham operation. This finding implies that there was a more potent stimulation of megakaryocyte maturation when platelet depletion was added to the nonspecific stimulus imposed by surgery alone. Other observations have shown that macromegakaryocytosis develops in response to thrombocytopenia.\(^{4,9,15}\) After sham operation, however, megakaryocyte size is normal at those times when it is most enlarged after exchange transfusion and platelet depletion.\(^{9}\) Therefore, it is likely that significant thrombocytopenia did not occur during sham operation, and that nonspecific thrombocytosis may be mediated only by turnover of increased numbers of megakaryocytes.

Failure of the megakaryocyte differential count to change in the presence of more rapid maturation suggested that, with onset of accelerated maturation, an increased number of cells began differentiating into the megakaryocyte compartment. Total numbers of megakaryocytes in the marrow were not estimated, so it could not be determined if a change occurred or if the increased influx balanced the shortening of \( T_{mk} \). Harker\(^{15}\) observed an increase in the absolute number of megakaryocytes with thrombocytopenia of four to ten days duration. For this accumulation of megakaryocytes to occur, the rate of influx into the stage I compartment must have exceeded the rate of destruction of stage III cells. In the presence of acceleration of maturation from stage I to III, this implies that differentiation of precursor cells into recognizable megakaryocytes was greatly increased. In those rats which were stimulated for four days, the number of megakaryocytes was increased by about one-third, indicating a relatively small daily increment which may not have been detectable in the differential counts in our studies. Alternatively, overproduction of stage I megakaryocytes may not occur with a single thrombocytopenic stimulus and may require more prolonged stress.

In contrast to the shortening of \( T_{mk} \) during recovery from thrombocytopenia is the normal maturation seen in the presence of sustained transfusion in-
duced thrombocytosis. The rate of maturation of megakaryocytes apparently can be stimulated by severe thrombocytopenia but not suppressed by sustained, moderate thrombocytosis. In the present studies, thrombocytosis developed during the recovery from thrombocytopenia. The subsequent decrease of platelet levels toward normal proceeded slowly and could have been mediated simply by resumption of normal megakaryocytopoiesis. Micromegakaryocytopoiesis may have developed in response to the reactive thrombocytosis, but there is no data on the size of individual megakaryocytes in this condition.

Passive transfer studies suggest that there is a humoral substance in the plasma of thrombocytopenic animals which stimulates platelet production. It might be suggested that this transferable factor in the plasma is responsible for increased thrombocytopoiesis in the thrombocytopenic animal. As was observed in thrombocytopenic animals, injection of thrombocytopenic plasma is followed by a delay of 2 days before platelet production apparently increases, suggesting that there is little delay between onset of thrombocytopenia and production of the active principle. The passive transfer studies were done with repeated injections of active serum which raises the possibility that the lag period of one day seen in the present studies before megakaryocyte maturation was accelerated may have been dependent on accumulation of a critical concentration of thrombopoietic activity. Alternatively, the major effect may be at the stem cell level with production of megakaryocytes with an inherent capability for more rapid maturation, although the data suggest that preexisting megakaryocytes are also affected.

Macromegakaryocytosis, also, has been demonstrated in thrombocytopenic rats and appears to be due to a feed-back which is active at the stem cell level. This hypertrophy of megakaryocytes is not dependent on prolonged thrombocytopenia, as it is demonstrable in stage I and II megakaryocytes 24 hours after a single thrombocytopenic episode. The coincidence of onset of acceleration of differentiation and maturation and the appearance of macromegakaryocytes about one day after platelet depletion suggests that the thrombocytopenic stimulus has two effects on megakaryocytic precursor cells. One causes the cells to differentiate more rapidly than normal, and the other produces macrocytosis. The same stimulus may mediate both effects by causing precursor cells to differentiate from a proliferating pool into the compartment of nondividing megakaryocytes during DNA synthesis or the pre-mitotic G2 period. Increased cellular DNA and size of megakaryocytes could then result from the missed mitoses of the precursor cells.

**SUMMARY**

Increased production of platelets was apparent during recovery from acute thrombocytopenia in rats. Preceding this there was acceleration of maturation of megakaryocytes as determined from the pattern of labeling with HThdR. Turnover of an increased number of megakaryocytes was indicated by maintenance of normal megakaryocyte differential counts in the presence of accelerated maturation.
**SUMMARIO IN INTERLINGUA**

Un augmento del production de plachettas esseva apparente in ratios durante lor restablimento ab thrombocytopenia acute. Le phenomeno esseva precedite de acceleration in le maturation de megakaryocytos secundo determinationes del configuration de marcage con TdR a tritium. Le facto que un augmentate numero de megakaryocytos transiva esseva demonstrate per le observation que le numeration differential de megakaryocytos remaneva normal in le presenta del un maturation accelerate.

**REFERENCES**

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