Induction of Thrombocytopenia by Thrombopheresis in Man: Patterns of Recovery in Normal Subjects During Ethanol Ingestion and Abstinence

By Louis W. Sullivan, William H. Adams, and Yong K. Liu

Using the technique of thrombopheresis (TP), platelet and megakaryocyte dynamics following acute thrombocytopenia were studied in two normal subjects during periods of ethanol ingestion and abstinence. Thrombocytopenia was induced over a period of 12 hr. A logarithmic decline in platelet count during TP and the serial morphologic changes in megakaryocytes during recovery from thrombocytopenia are described. Although these parameters were not affected by ethanol ingestion, platelet counts after TP did not return to normal until ethanol was discontinued. $^{51}$Cr-labeled platelet survival was normal in one subject studied, and no evidence of increased platelet sequestration was found. It is concluded that heavy ethanol ingestion induces, augments, or sustains thrombocytopenia by impairing megakaryocytopoiesis in man. The mechanism by which ethanol induces thrombocytopenia may be, in part, to "ineffective thrombopoiesis," impairment of the differentiation of precursor cells into the megakaryocytic compartment, or a combination of these factors.

A CUTE THROMBOCYTOPENIA has been induced in animals by injection of antiplatelet serum, or by exchange transfusion with platelet-poor blood. $^1$ $^9$ Thrombocytopenia has been observed in patients who have received massive transfusions of whole blood, and has been induced in normal individuals by infusion of plasma from subjects with idiopathic thrombocytopenic purpura. $^{10}$ $^{13}$ Only Shulman et al., however, have described the response to acute thrombocytopenia produced by "plasmapheresis" in normal and asplenic man, but details of their methods have not been presented. $^{14}$ The present study uses the similar technique of thrombopheresis (TP) to determine not only the rate and pattern of recovery of circulating platelets, but also serial changes in the morphology of marrow megakaryocytes following acute thrombocytopenia in two normal subjects. In addition, the same subjects have served as their own controls in a study of the effect of ethanol on thrombopoiesis after TP. The results indicate that ethanol produces thrombocytopenia by impairing platelet production and/or shedding.
MATERIALS AND METHODS

Subjects

Two 43-yr-old healthy white males (E.W. and R.K.) were studied in the Clinical Research Unit of the Boston University Medical Service at Boston City Hospital. (These two subjects and a third control subject gave their informed consent.) Both had histories of intermittently excessive ethanol intake. However, physical examinations, tests of liver function (including serum SGOT, lactic acid dehydrogenase, albumin, globulin, bilirubin, alkaline phosphatase, BSP retention, and one-stage prothrombin time), serum electrolytes, blood urea nitrogen, serum creatinine, calcium and phosphorus, stool guaiac tests, electrocardiograms, and x-rays of the chest and abdomen were normal prior to admission. Although control values for platelet survival were not obtained, it should be stressed that each subject served as his own control in this study. Both subjects were on the regular hospital diet, and the only medications received were secobarbital (100 mg at bedtime for sedation) and paraaldehyde, after sustained periods of ethanol ingestion.

Procedures

Daily hematocrits, reticulocyte counts, and total differential leukocyte counts were performed according to standard methods. Platelet counts were done on venous blood by phase microscopy. Coded bone marrow smears from the sternum or iliac crest were stained with Wright-Giemsa solution, and differential counts were performed on 200 or more consecutive marrow megakaryocytes. These were grouped into three classes, modified from the morphologic criteria of Bessis: (1) megakaryoblasts, (2) basophilic megakaryocytes, and (3) mature megakaryocytes (granular megakaryocytes, platelet-producing megakaryocytes, and naked megakaryocytic nuclei). Smears from each marrow specimen were also fixed before drying (Spraycyte, Clay-Adams, Parsippany, N.J.) and stained by the Papanicolau method for better visualization of megakaryocyte lobes. The results were recorded as (1) the number of lobes recognized per cell (observed number) and (2) a “derived” lobe count, an exponential factor of 2 (e.g., if 13 lobes were counted, the derived lobe number was 16). The number of megakaryocytes per 10,000 nucleated marrow cells was also counted on coded slides.

Coagulation studies included a one-stage prothrombin time, activated partial thromboplastin time, thrombin time, and fibrinogen determination. A minimum estimate of the splenic platelet pool was obtained by intravenous infusion of epinephrine at a dose of 5 μg/kg body weight over a 20-min period. Platelet survival was measured in R.K. with 51Cr-labeled autologous platelets 3 days before his TP, and platelet specific activity was followed during, and for 3 days after induction of thrombocytopenia. Plasma volume was determined in E.W. using Evans blue dye before and at the conclusion of TP. Serum vitamin B12 levels were determined by a radioisotope dilution method and serum folate was assayed microbiologically with Lactobacillus casei.

For TP, venous blood was collected into double plasmapheresis bags containing 75 ml of acid-citrate-dextrose solution (USP formula A: double plasmapheresis Blood-packs, PA-220, and AE-2 Transfer Tubing, Fenwall Labs, Morton Grove, Ill.) and centrifuged at 600 g for 10 min at room temperature. The platelet-rich plasma was removed and centrifuged at 1500 g for 30 min, and the resulting platelet button discarded. The platelet-poor plasma was then mixed with the subject’s packed erythrocytes and reinfused. With this technique 17–19 units of blood were exchanged in 12 hr. Platelets were counted after removal of every 2 units of blood and coagulation tests were performed after removal of every 4 units. In R.K., additional 3-unit TP were performed 24 hr after the initial procedures in an attempt to lower the platelet count to the same absolute value as in E.W.

To assess whether any platelet injury secondary to this method of mechanical removal of platelets results in thrombocytopenia because of possible release of substances from damaged platelets into reinfused blood, an identical procedure was performed in a third normal subject. Harvested platelets were reconstituted with autologous plasma and red cells prior to reinfusion, and platelet counts were obtained during and for 10 days after this “sham” TP.

In the second phase of the study, ingestion of ethanol, in the form of 86 proof whiskey (Imperial Whiskey, Hiram Walker and Sons, Peoria, Ill.), was begun 6 days prior to TP and continued for a total of 24 days (E.W.) and 21 days (R.K.). The average daily doses were 380 g of
ethanol (36 oz whiskey) and 284 g of ethanol (27 oz whiskey), respectively, consumed in divided doses over an 18-hr period at 2-hr intervals from 6 a.m. to 12 p.m. Blood ethanol levels were determined between 6 p.m. and 8 p.m. and at the beginning of TP.

RESULTS

Control period. Both subjects had stable blood counts, including platelet counts, during the control period before TP. Daily platelet counts averaged $306 \times 10^9$/liter in E.W. (range 275–326) and $379 \times 10^9$/liter in R.K. (range 343–395). (See Fig. 1.) With epinephrine infusion, platelet counts rose 410, and

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Fig. 1. Recovery from TP-induced thrombocytopenia. Results of megakaryocyte differential counts are shown in upper histogram. Shaded area is approximate range of "normal" platelet count in man.16 (A) For subject E.W., the recovery rate is compared to predicted rates based on either random or senescent platelet destruction (see text). (B) A 3-unit TP was performed 24 hr after the initial one in R.K. (small arrow).
23°, above baseline counts in E.W. and R.K., respectively. Coagulation studies were normal. Serum vitamin B₁₂ levels were 388 and 264 pg/ml, and serum folate levels were 13 and 15 ng/ml, respectively.

Induction of thrombocytopenia. TP of 19 and 17 units were performed in E.W. and R.K., respectively, while they were not ingesting ethanol. At the conclusion of this procedure the platelet counts were 28°, of the initial values in both subjects (75 × 10⁹/liter in E.W. and 102 × 10⁹/liter in R.K.; see Fig. 1). Serial coagulation studies during TP remained normal. The decrease in the concentration of circulating platelets during TP was logarithmic in both subjects. The specific activity of ⁵¹Cr-labeled autologous platelets was constant during TP of R.K. There was no significant change in platelet count in the third subject during or after the procedure of sham TP of 18 units.

TP of 18 units (E.W.) and 19 units (R.K.) were again performed on the sixth day of ethanol ingestion. Blood ethanol levels on the day of TP were 220 mg/dl and 214 mg/dl, respectively. Platelet counts at the end of TP were 70 × 10⁹/liter in E.W. (16°, of initial value) and 86 × 10⁹/liter in R.K. (30°, of initial value). (See Fig. 2.)

Recovery from TP-induced thrombocytopenia. The rate of return to normal platelet counts in the two subjects during abstinence is shown in Fig. 1. The recovery rate of E.W. is compared to a theoretical recovery rate derived by assuming (1) a mean platelet life span of 10 days, (2) a stable basal rate of platelet production (the daily rate being calculated as one-tenth the baseline platelet count), and (3) either random or senescent platelet destruction. The average daily increase in platelets for the first 84 hr after TP was 23.5 × 10⁹/liter. Thereafter, the platelet count rose more rapidly in both subjects, averaging 70 × 10⁹/liter (E.W.) and 65 × 10⁹/liter (R.K.) per day for 4 5 days. Following a transient plateau of platelet counts in both subjects on the eighth day, a rise in platelets resumed thereafter at a similar rate. Eventually, thrombo-

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**Fig. 2.** Recovery from TP-induced thrombocytopenia during ethanol ingestion. Quantity of ethanol ingested is given in lower histogram. Shaded area is approximate range of "normal" platelet count in man.° (A) Results in subject E.W. (B) A 3-unit TP was performed 24 hr after the initial one in subject R.K.


Table 1. Nuclear Lobe Counts per 100 Megakaryocytes During Recovery From TP-induced Thrombocytopenia

<table>
<thead>
<tr>
<th>Time Observed</th>
<th>Derived*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
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<tr>
<td>Subject E.W.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>688</td>
</tr>
<tr>
<td>24 hr after TP</td>
<td>604</td>
</tr>
<tr>
<td>72 hr after TP</td>
<td>800</td>
</tr>
<tr>
<td>144 hr after TP</td>
<td>685</td>
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<tr>
<td>Subject R.K.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>716</td>
</tr>
<tr>
<td>24 Hours after TP</td>
<td>578</td>
</tr>
<tr>
<td>72 Hours after TP</td>
<td>950</td>
</tr>
<tr>
<td>120 Hours after TP</td>
<td>736</td>
</tr>
<tr>
<td>192 Hours after TP</td>
<td>723</td>
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</table>

*Derived values are expressed as percentage of megakaryocytes in each of the five lobe-number categories, an exponential factor of 2.19
†Statistical analysis was performed using a 3 × 2 × 2 *test* in which 2- and 4-lobe and 16- and 32-lobe values were grouped, and each time period was compared to control. The significance of the shift in lobe values is a function of the relative changes among the three groups. The difference between 24- and 72-hr specimens was highly significant in both subjects (p < 0.001). Also pertinent is the obvious similarity in shifts noted in both subjects. NS: not significant.

cytosis was observed in both subjects on the eleventh day. Epinephrine infusions at 24 hr (R.K.) and at 48 hr (E.W.) after TP resulted in transient increases in platelet count by 24% and 61%, respectively.

In contrast, the ingestion of ethanol was associated with a delayed and incomplete recovery of platelet counts (Fig. 2). Rises in platelet count during epinephrine infusions in E.W. 24 and 48 hr after TP were 24% and 15%, respectively. 51Cr-labeled platelet survival in R. K., begun on the third day of ethanol ingestion and continued for 3 days after TP, was 8 days, a normal value.22

Bone marrow changes. Before TP, megakaryocyte differential counts indicated that there were 14%, (E.W.) and 20%, (R.K.) “immature megakaryocytes” (basophilic megakaryocytes and megakaryoblasts) (Fig. 1). The proportions of immature megakaryocytes after TP were 25%, 11%, at 24 hr, 35%, at 48 hr, and 15%, and 30%, at 72 hr, respectively. In both subjects the number of observed nuclear lobes in 100 megakaryocytes 24 hr after TP was less than control; at 72 hr it was greater than control, and the number returned to control levels by 120 hr in R.K. and 144 hr in E.W. (Table 1). Temporal changes in the total derived lobe count expectedly paralleled the observed count. The distribution of the derived lobe count showed an increased number of megakaryocytes with 2 or 4 lobes 24 hr after TP. At 72 hr there was a decrease in the per cent of 2- or 4-lobed cells and an increase in the per cent of 8-, 16- and 32-lobed megakaryocytes. The distribution approached original values by 120–144 hr. Twenty-four hours after TP during ethanol ingestion there was an increased percentage of megakaryoblasts and basophilic megakaryocytes in the marrow of both subjects (Fig. 3). A smaller number of nuclear lobes per 100 megakaryocytes was also noted (R.K., Table 2; specimen from E.W. was technically unsatisfactory). In marrows obtained during ethanol ingestion 16 days (E.W.)
and 9 days (R.K.) after TP, there was an increased number of nuclear lobes in the megakaryocytes. Vacuolization of erythroblasts was noted after 6 days of ethanol ingestion (i.e., on the day of thrombopheresis). Although occasional macronormoblasts were found, marrow morphology was predominantly normoblastic. Marrows obtained after 15 and 24 days of ethanol ingestion had an increased number of macronormoblasts and occasional megaloblasts. There were no changes detected in the number of megakaryocytes per 10,000 nucleated marrow cells after TP during abstinence or ethanol ingestion.

At a later period, when E.W. had a normal hemogram and stable platelet count, he was given 212–252 g of ethanol (20–24 oz whiskey) daily for 18 days (Fig. 4). During this period his platelet count decreased by 26% (from 306 to 225 × 10^9/liter). The percentage of megakaryoblasts and basophilic megakaryocytes present in the marrow after 15 days of ethanol ingestion was 14%, a value comparable to that previously found in this subject when not drinking. After cessation of ethanol the percentage of immature megakaryocytes was 19%, at 12 hr, 32%, at 36 hr, and 31% at 60 hr. The platelet count did not increase significantly for 60 hr after abstinence. The rate of platelet increase for the next 2 days was 33.5 × 10^9/liter, then rose to 67 × 10^9/liter/day between the sixth through eighth postethanol days (Fig. 4).

Table 2. Nuclear Lobe Counts per 100 Megakaryocytes During Recovery From TP-induced Thrombocytopenia During Ethanol Ingestion

<table>
<thead>
<tr>
<th>Time Observed</th>
<th>Derived</th>
<th>Total</th>
<th>p t</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Derived</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 4 8 16</td>
<td>31 4</td>
<td></td>
</tr>
<tr>
<td>Subject E.W.</td>
<td>Control</td>
<td>714 6 18 52 24 0</td>
<td>884</td>
</tr>
<tr>
<td>16 days after TP</td>
<td>845 3 16 46 31 4</td>
<td>1062</td>
<td></td>
</tr>
<tr>
<td>Subject R.K.</td>
<td>Control</td>
<td>698 4 16 60 20 0</td>
<td>872</td>
</tr>
<tr>
<td>1 day after TP</td>
<td>617 11 22 50 17 0</td>
<td>782</td>
<td></td>
</tr>
<tr>
<td>9 days after TP</td>
<td>934 3 18 44 27 8</td>
<td>1118</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*Derived values are expressed as percentage of megakaryocytes in each of the five lobe-number categories, an exponential factor of 2.19

†See Table 1 for method of statistical analysis.
DISCUSSION

Our observations on platelet and megakaryocyte dynamics in response to thrombocytopenia produced by TP in man can be divided into three phases; induction, early recovery, and late recovery.

Induction of thrombocytopenia was similar whether or not the subjects were ingesting ethanol. The decrease in platelet count was logarithmic and equivalent degrees of thrombocytopenia were achieved in all instances. This logarithmic decline in platelets has been observed in rats made thrombocytopenic by exchange transfusion3,9 and in man after plasmapheresis.14 Using 51Cr-labeled platelets Shulman et al. showed a normal rate of decline in specific activity before and after induction of thrombocytopenia.14 Similar results were obtained in the present study. In addition, no significant alteration of 51Cr specific activity was noted during the process of TP. Jackson et al. have reported that citrate does not contribute to the development of thrombocytopenia in dogs receiving platelet-poor autologous blood.2 We likewise found that citrate has no such effect in man, because the procedure of sham TP did not significantly alter the platelet count in our control subject during or after TP.

The early recovery from TP showed the following sequence of events: a steady-state rise in platelet count for 84 hr after TP, during which time an increased percentage of young megakaryocytes was noted at 24 hr and increased lobulation of megakaryocytes at 72 hr. The steady-state increase in platelet count merits comment. Shulman et al. induced thrombocytopenia by plasmapheresis in six normal and three splenectomized subjects.26 The three asplenic
subjects and one of the normal subjects showed a rise in platelet count within 24 hr after the induction of thrombocytopenia, whereas the remaining normal subjects exhibited no increase in platelet count during this period, suggesting splenic sequestration of young platelets. However, studies in rats made thrombocytopenic by exchange transfusion showed a normal rate of increase in platelet counts for the first 2 days, followed by an accelerated rise in platelets and a rebound thrombocytosis. Similar findings were obtained in the present study. Epinephrine infusion 24 and 48 hr after the induction of thrombocytopenia did not result in a percentage increase in platelet count significantly greater than control values in either subject. These findings are consistent with observations in rats in which no preferential splenic sequestration of young $^3$H-diisopropyl fluorophosphate-labeled platelets was detected. One possible explanation for the difference in the rate of early platelet increase we observed, which was in contrast to Shulman’s observations, may be the degree of thrombocytopenia produced. With the lesser degrees of thrombocytopenia achieved by Shulman et al. slight increases in platelet count may have been less discernible than in the present study, where more severe thrombocytopenia was induced. A more likely explanation may be found in animal studies showing that platelet production increases proportionately with the degree of thrombocytopenia.

In contrast to the above, early recovery after TP was impaired during ethanol ingestion. The development of sustained thrombocytopenia in folate-deficient subjects while ingesting 156 g of ethanol daily has been previously reported. In the present studies of healthy and initially non-folate-deficient subjects, ethanol ingestion of only 6 days duration impaired the recovery from acute thrombocytopenia induced by TP. Folic acid supplementation was not given during this period, although a regular hospital diet was provided. It is possible that some degree of folate depletion occurred as a consequence of anorexia, although it is unlikely that dietary deficiency, per se, was of sufficient severity in these relatively acute studies to have impaired hemopoiesis. The survival of autologous $^{51}$Cr-labeled platelets in one subject was normal. A shortened platelet life span has been reported to be one cause of alcohol-induced thrombocytopenia (although artifact due to labeling technique is of concern). The present study does not support this postulated mechanism. Coagulation studies during and after TP were normal and epinephrine infusion gave no evidence of increased splenic sequestration of platelets. Thus impairment of recovery of the platelet count in these two subjects during the early phase of ethanol ingestion appeared to be due to impaired platelet production. This direct toxic effect on the marrow has been postulated previously. During late recovery from TP, a three-fold increase in the rate of rise in platelet count was observed beginning 84 hr after TP during abstinence. The morphologic changes observed in megakaryocytes during the early recovery phase presumably accounted for this increase. Cronkite et al. have presented evidence that in man the normal maturation time of megakaryocytes from stem cell to the appearance of platelets in the circulation is 6–10 days. Our observations suggest that in subjects made acutely thrombocytopenic, changes
in the megakaryocyte compartment may result in accelerated platelet production and shedding after only 84 hr. The accelerated rate of platelet production observed in our thrombocytopenic subjects after 84 hr is compatible with a more rapid transit through the megakaryocytic compartment. Ebbe et al. noted that when 3H-thymidine was injected into rats 24 hr after induction of thrombocytopenia, the appearance of this isotope in mature megakaryocytes was more rapid than in normal rats.5 With thrombocytopenia, however, an accelerated influx of cells from precursor pool into the megakaryocytic compartment may occur, since Ebbe et al. found an increased labeling index in young megakaryocytes in their animals.5 It is also possible that the stimulus of acute thrombocytopenia may have induced a cohort of young megakaryocytes to develop increased nuclear ploidy with production of a greater number of platelets per megakaryocyte, accounting for (or contributing to) the increased rate of platelet production. Evidence has been presented that an increase in average megakaryocyte size and nuclear segmentation is associated with an increased number of platelets produced per megakaryocyte.19,35 Increased nuclear lobulation of megakaryocytes was observed in our subjects 72 hr after induction of thrombocytopenia, but disappeared 6 days after TP. (A more accurate assessment of ploidy would require determinations of megakaryocyte DNA content.36) Therefore evidence of an increased rate of platelet production between days 6 and 11 may have been due to an increase in megakaryocyte number rather than increased size of megakaryocytes. Indeed, it is possible that the former event could have explained the increased rate of platelet production noted at 84 hr, because the time required for greater nuclear ploidy to develop may actually prolong maturation time. It has been suggested that an increase in megakaryocyte number occurs in rats with sustained thrombocytopenia.7,37 By simple counting techniques we were unable to detect an increase in the number of megakaryocytes per 10,000 nucleated marrow cells after TP.

A transient plateau in the rate of platelet production was observed in both subjects 8 days after TP. The cellular kinetics responsible for this phenomenon remain unclear. Further observations are necessary to determine if this is a consistent event, and therefore suggestive of periodicity in response.

In contrast to the above normal late recovery pattern, ethanol ingestion prevented a return to normal platelet count until it was discontinued. Of interest, the percentage of megakaryoblasts and basophilic megakaryocytes in the marrow increased transiently after TP (Fig. 3), comparable to changes during abstinence, and subsequently increased nuclear lobulation of megakaryocytes after TP was not accompanied by a rise in platelet counts to control values. This finding suggests that ingestion of ethanol may cause "ineffective thrombopoiesis;" i.e., apparently stimulated megakaryocytosis did not result in increased platelet production and/or shedding. "Ineffective thrombopoiesis" has been reported in the DiGuglielmo syndrome, in a syndrome of familial thrombocytopenia, and in megaloblastic anemia.19 After TP, no significant change was detected in the number of megakaryocytes per 10,000 nucleated marrow cells. However, these observations do not measure total marrow cellularity. More quantitative techniques for measuring megakaryocyte
mass and number are necessary before normal and ethanol-suppressed recovery kinetics of megakaryocytes can be completely evaluated.

After cessation of ethanol in E.W. (Fig. 4) there was no significant rise in platelet count for 3 days. Platelet production then increased sixfold. This interval was similar to the time required for augmented platelet production to occur after TP during abstinence. This observation suggests that new platelets entering the circulation 3 days after cessation of ethanol were progeny of cells which were immature megakaryocytes (or their precursors) at the time of cessation of ethanol ingestion. Another possibility was that this 3-day period was necessary for functional recovery of existing marrow megakaryocytes from the effects of ethanol on platelet production or shedding. A third possibility is that ethanol may have impaired differentiation of stem cells into the megakaryocytic compartment.

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