Platelet Production in Experimental Iron Deficiency Anemia

By Sun I Choi and Joseph V. Simone

Abnormal platelet counts have been observed in patients with iron deficiency anemia. To study this relationship, rats were made iron deficient, and platelet production was estimated by radiosulfate incorporation into platelets. With progressive iron deficiency anemia, both platelet counts and the rate of platelet production increased significantly. Platelet survival was normal. Injection of iron was followed by a fall in platelet counts and platelet production to normal levels within 72 hr, at which time stainable iron had appeared in the bone marrow. An inverse relationship between platelet counts and hematocrit was also seen. It appears that platelet homeostasis in iron deficiency anemia is influenced by the duration and severity of anemia.

THE ASSOCIATION of thrombocytosis with iron deficiency anemia has been documented both in children[1] and in adults.[2] Thrombocytopenia may also occur in severe cases of iron deficiency anemia.[3] In both conditions, the platelet counts returned to normal after iron therapy. The mechanism of these changes in platelet levels is not known. The present study was undertaken to investigate the relationship of platelet production to iron deficiency anemia in rats.

MATERIALS AND METHODS

Male, Sprague-Dawley rats weighing 100-150 g were made iron deficient either by initially bleeding up to 20% of blood volume followed by a low-iron diet (Nutritional Biochemicals Co., Cleveland, Ohio), or by low-iron diet alone without initial bleeding. At weekly intervals, animals were weighed and blood samples were obtained by tail cut for determining hemoglobin levels (cyanmethemoglobin method) and platelet counts (phase-contrast microscopy). The experiments were performed after at least 4 wk of a low-iron diet. An exception is the experiment in which platelet production was studied in five of 40 animals each week after starting the low-iron diet.

Measurement of Platelet Production

Platelet production was measured using a modification of the method of Odell et al.[4] Injected radiosulfate ($Na_2{}^{35}SO_4$) is chemically incorporated into megakaryocyte cytoplasm and remains fixed and nonexchangeable throughout the formation, release, and circulating lifespan of platelets.[5] The isotope is not incorporated into circulating platelets; therefore, only newly circulating platelets are labeled. One microcurie of sodium sulfate ($Na_2{}^{35}SO_4$) per gram of body weight was given intravenously. Twenty-four hours later, 5.0 ml of blood were drawn from the vena cava under ether anesthesia into an EDTA-wetted plastic syringe and were placed in a plastic

From the Laboratory of Hematology, St. Jude Children's Research Hospital, Memphis, Tenn. 38101.

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Sun I Choi, M.D.: *Assistant Member in Hematology, St. Jude Children's Research Hospital, Memphis, Tenn. 38101.* Joseph Simone, M.D.: *Chief of Hematology, St. Jude Children's Research Hospital, Memphis, Tenn. 38101.*

tube containing five drops of Sequester-Sol (K_2-EDTA). Platelets were counted and then were separated from each sample by adding 3.0 ml of isotonic saline, by centrifuging at 121 *g* for 20 min at 4°C, and by removing the platelet-rich supernatant. Three milliliters of isotonic EDTA-saline were added to the original tube, and the packed cells were resuspended and recentrifuged. This procedure was repeated once more, and the three supernatants were pooled. With this technique, 90–95% of the platelets in the original sample were recovered. The pooled, platelet-rich supernatant was centrifuged at 1475 *g* for 20 min to obtain a platelet button. The button was suspended in 1.0 ml of 1% ammonium oxalate for 10 min to lyse any remaining red cells. The platelets were then washed twice with 7.0 ml of EDTA-saline. The supernatant fluid from these washes contained less than 2% of the sample radioactivity. After the final wash, the platelet button was suspended in 1.2 ml of isotonic saline. This final suspension contained only 0–2 white blood cells per 10^6 platelets and was free of red cells. To 1.0 ml of the final platelet suspension, 3.0 ml of 5% trichloroacetic acid solution and 500 mg of bovine serum albumin (BSA) were added to separate protein-bound ^{35}S from free sulfate. The mixture was cooled at 4°C for 15 min, and was centrifuged at 700 *g* for 10 min; the precipitate was drained well. The discarded supernatant was essentially free of radioactivity. One milliliter of NCS solvent (Amersham-Searle, Des Plaines, Ill.) was added to the precipitate yielding a clear sample after mixing. Samples were transferred to counting vials, and 10 ml of scintillation liquid (Liquiflour, New England Nuclear Corp., Boston, Mass.) were added. A blank vial contained 1.0 ml of NCS and 10 ml of Liquifluor. Sample radioactivity was determined using a Packard Tri-Carb Liquid Scintillation Spectrometer with an internal standard. Counting efficiency for radiosulafte was calculated to be 85%. Background radioactivity ranged from 15 to 25 cpm.

Relationship of Platelet Production to Degree of Iron Deficiency Anemia

This study included 40 rats given a low-iron diet alone without initial bleeding and 40 controls given a normal diet. At weekly intervals, platelet radioactivity was determined in five rats from each group 24 hr after injection of radiosulfate. Hemoglobin, reticulocyte count, and platelet count were determined weekly.

Platelet Production After Iron Injection

The mean ±1 SD of the hemoglobin level in 12 normal rats in this study was 14.6 ± 0.8 g/100 ml. After 5 wk of low-iron diet, 12 (100/g) rats had hemoglobin levels of 5.3–7.9 g/100 ml. Iron dextran (Imferon), 1.5 mg intraperitoneally, was given to each rat in the iron-deficient and control groups. Hemoglobin, reticulocyte count, and platelet count were determined before injection of iron and at the time of sacrifice, when blood samples were obtained for measuring platelet production 24, 48, and 72 hr after iron injection. Radiosulfate was given 24 hr prior to sacrifice. Bone marrow smears were made and stained for hemosiderin.

Platelet-Hematocrit Relationship

Four groups of four rats each were used in this experiment: normal controls, iron-deficient rats, iron-deficient rats transfused with homologous normal red cells, and iron-deficient rats transfused with homologous iron-deficient red cells. Iron-deficient rats had hemoglobin levels of 5.9–9.1 g/100 ml before transfusion. Packed red cells for transfusion were washed once with isotonic saline, and 2.0 ml were given intraperitoneally on three consecutive days. After three transfusions, mean hemoglobin levels had risen to 12.5 g/100 ml, and hematocrits rose to 40% in both transfused groups. Radiosulfate was injected on the fourth day, and samples were collected 24 hr later. Because of a subjective impression that megakaryocyte numbers in bone marrow differed, for this study only numbers were estimated from marrow smears by counting cells in 100 high-power fields.

Platelet Survival

Platelet survival was determined in iron-deficient and control rats using a modification of the radiosulfate method described by Odell and Anderson.[6] Radiosulfate, 145 μCi on the first day and 100 μCi daily for 8 days, was given to each animal. Two rats from each group were sacrificed daily, and platelet radioactivity was determined by the method described above. Platelet survival in days is the time to maximum platelet labeling.

RESULTS

Platelet Count in Iron Deficiency Anemia

The effect of progressive iron deficiency produced by initial bleeding and a low-iron diet is shown in Fig. 1. Platelet counts had risen markedly by 7 days after bleeding and fell to an intermediate level by 2 wk. Thereafter, the platelet counts steadily increased with progressive anemia. Rats given a low-iron diet without prior bleeding developed progressive anemia and ultimately showed a similar degree of anemia by the fourth week. Platelet counts changed little in

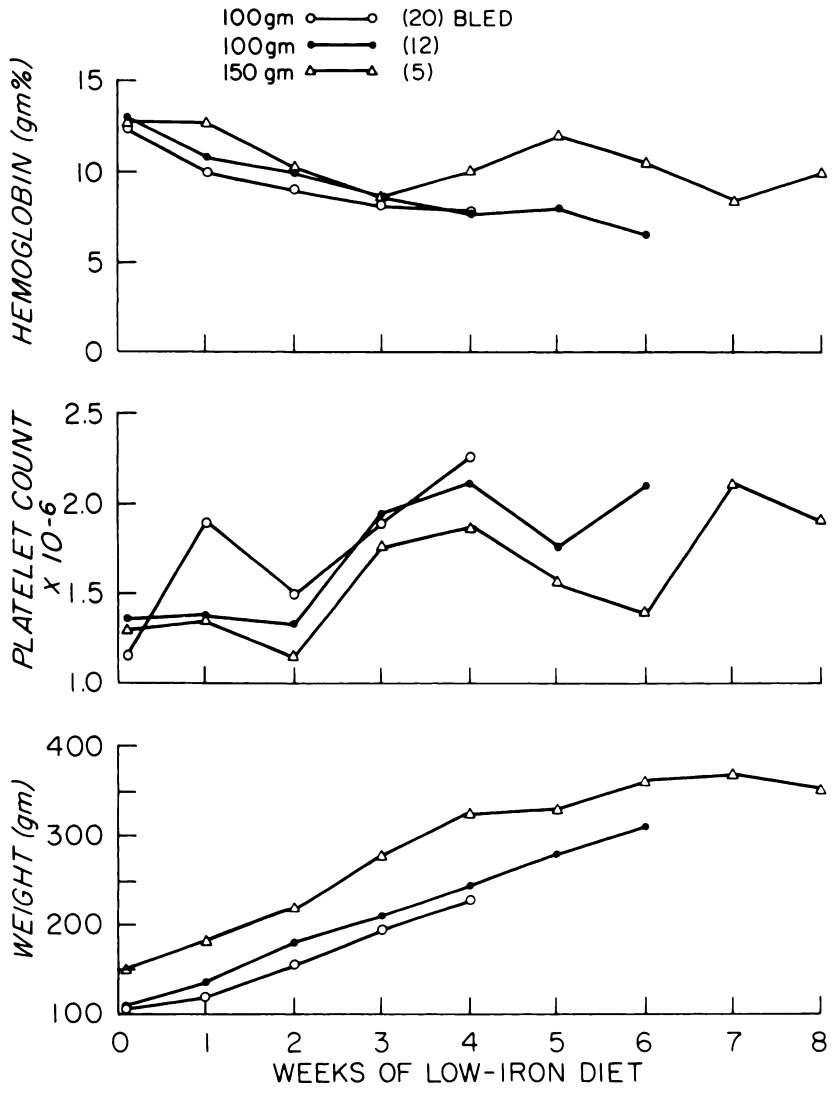

Fig. 1. Longitudinal study of rats on a low-iron diet. Initial weight was 100 or 150 g. One group of 20 rats was bled at the start of low-iron diet. Numbers in parentheses represent the numbers of rats in each group.

the first 2 wk, rose between the second and third weeks, and stabilized at that level for the remainder of the experiment. Results in the third group of rats, which were older and heavier (150 g) when started on the low-iron diet, were essentially the same. Growth rates were similar in all three groups. The iron-deficient animals weighed 30–40 g less than controls, but they appeared healthy and active. The commercially available low-iron diet was sufficient to produce anemia in the smaller animals without initial bleeding. Predictably, iron deficiency anemia was easier to induce and was greater in degree in younger rats. Since animals were neither given a totally iron-free diet nor placed in an iron-free environment, they may have ingested trace amounts of iron from sources such as tap water or cages. Platelet counts increased after a latent period of 2 wk and reamined high as the anemia progressed, and in no instance did thrombocytopenia develop during the period of observation.

Platelet Production

Four to 5 wk after initial bleeding and the start of a low-iron diet, the rate of platelet production was estimated by radiosulfate incorporation into platelets. The results of four experiments are shown in Table 1. Radiosulfate incorporation was significantly greater in iron-deficient animals than in controls ($p < 0.05$). Although absolute radioactivity of platelets varied between experiments, there was no overlap between control and anemic groups, and average radiosulfate incorporation was two to three times greater in iron-deficient rats. As seen in Table 1, platelet counts in these iron-deficient rats did not differ greatly from controls, particularly when compared to the difference in radiosulfate incorporation.

Degree of Anemia

Since animals developed anemia at differing rates, all simultaneous determinations of hemoglobin and radiosulfate incorporation obtained in iron-deficient animals during these studies were pooled, and the results are shown in Table 2. Analysis of radiosulfate incorporation with hemoglobin levels yielded a correlation coefficient of -0.49 ($p < 0.001$). All simultaneous determinations of hemoglobin and platelet count were pooled and plotted in Fig. 2. A total of 496 determinations included normal controls and animals on a low-iron diet.

Table 1. Platelet Production in Iron Deficiency Anemia in Rats

Experiment No.		No. Rats	Hemoglobin (g/100 ml)	Platelet Count ($\times 10^6$)	^{35}S-Platelet (cpm/10^9 Platelets)
1	Control	4	14.9 ± 0.5	1.29 ± 0.2	1039
	Iron deficient	5	6.0 ± 1.4	1.72 ± 0.2	2076
2	Control	4	14.7 ± 0.4	1.03 ± 0.1	254
	Iron deficient	5	6.7 ± 0.9	1.70 ± 0.2	660
3	Control	4	14.9 ± 0.8	0.95 ± 0.2	130
	Iron deficient	5	5.9 ± 0.5	1.97 ± 0.3	248
4	Control	4	14.1 ± 1.6	1.02 ± 0.1	295
	Iron deficient	4	7.1 ± 0.2	2.09 ± 0.3	928

Each value represents mean ± 1 SD.

Table 2. Relationship of Hemoglobin Level and 24-Hr Radiosulfate Incorporation Into Platelets

Experiment No.		No. Rats	Hemoglobin (g/100 ml)	Platelet Count ($\times 10^6$)	^{35}S-Platelet (cpm/10^9 Platelets)	Ratio of Control/Experimental
1	Control	5	13.5 ± 0.5	1.45 ± 0.2	423 ± 44	
	Experimental	5	12.8 ± 0.6	1.37 ± 0.3	638 ± 102	1.5
2	Control	5	16.1 ± 0.6	1.22 ± 0.1	346 ± 19	
	Experimental	5	12.1 ± 0.5	1.53 ± 0.2	780 ± 76	2.2
3	Control	5	13.9 ± 0.1	1.11 ± 0.1	433 ± 27	
	Experimental	5	10.2 ± 0.2	1.30 ± 0.2	738 ± 85	1.7
4	Control	5	16.1 ± 0.4	1.11 ± 0.3	342 ± 69	
	Experimental	5	9.9 ± 0.9	1.92 ± 0.6	705 ± 164	2.1
5	Control	5	14.0 ± 0.3	1.22 ± 0.1	386 ± 24	
	Experimental	5	8.7 ± 0.2	2.09 ± 0.4	961 ± 137	2.5
6	Control	4	14.9 ± 0.8	1.21 ± 0.3	362 ± 67	
	Experimental	5	8.5 ± 0.4	1.78 ± 0.5	634 ± 133	1.7
7	Control	4	14.1 ± 1.6	1.02 ± 0.1	295 ± 20	
	Experimental	4	7.1 ± 0.2	2.09 ± 0.3	928 ± 99	3.1
8	Control	4	14.7 ± 0.4	1.03 ± 0.1	254 ± 19	
	Experimental	4	6.7 ± 0.9	1.70 ± 0.2	660 ± 58	2.6
9	Control	4	14.9 ± 0.8	0.95 ± 0.2	130 ± 20	
	Experimental	5	5.9 ± 0.5	1.97 ± 0.3	248 ± 28	1.9

Each value represents mean ± 1 SD.

Although platelet counts varied, progressively higher counts were seen as hemoglobin levels fell from 11 to 7.0 g/100 ml. Below 7.0 g/100 ml, platelet counts did not rise further and in fact declined toward normal or slightly above normal levels. Analysis of hemoglobin levels with platelet counts in Fig. 2 yielded a correlation coefficient of -0.37 ($p < 0.001$). Thus, the inverse correlation of hemoglobin levels with both radiosulfate incorporation and platelet counts were highly significant.

Platelet Production Following Iron Injection

Since a similar degree of anemia could be obtained after 4 wk of low-iron diet alone, animals were not bled initially in this study. Hematologic changes and platelet radiosulfate incorporation in iron-deficient animals after injection of iron are shown in Table 3. Twenty-four hours after iron, radiosulfate incorporation was 2.6 times control values. After 48 hr, radiosulfate incorporation dropped to 2.1 times control, and platelet counts were lower, but hemoglobin and reticulocyte levels had not changed. However, 72 hr after iron, reticulocyte counts had increased to double original levels, the mean hemoglobin increased from 6.3 to 9.5 g/100 ml, and both platelet counts and radiosulfate incorporation had returned to control levels. Reticulocytes must have increased between 48 and 72 hr to account for the rise in hemoglobin. Bone marrow smears showed no stainable iron until 72 hr after iron injection.

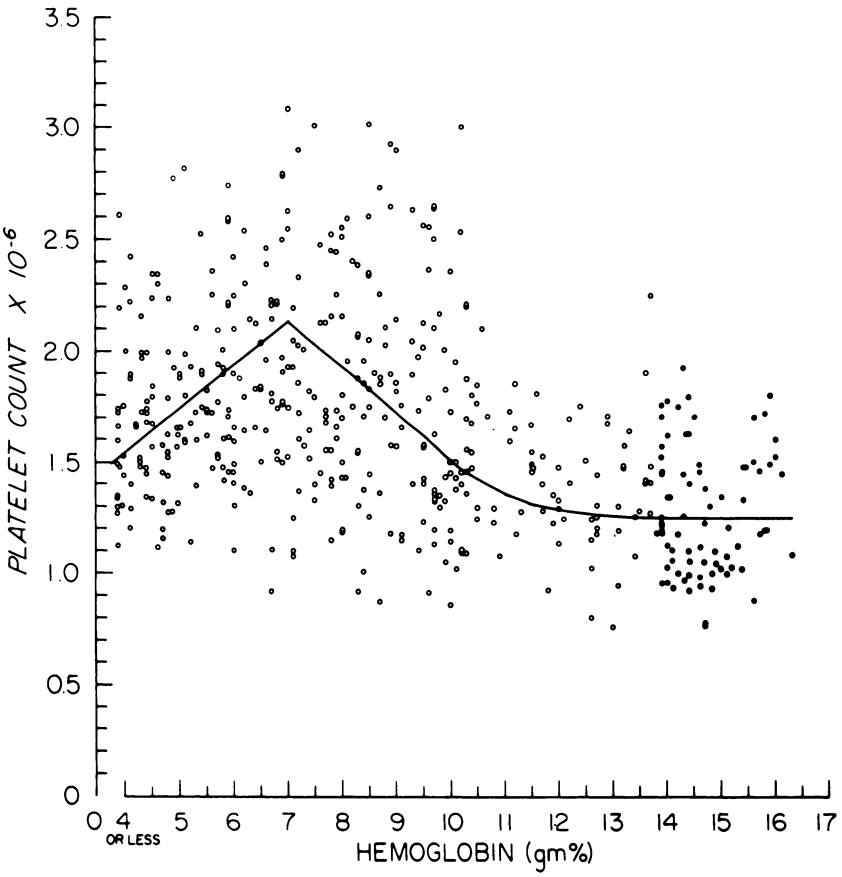

Fig. 2. Correlation of 496 simultaneous platelet counts and hemoglobins in normal and iron-deficient rats. Progressively higher counts were seen as hemoglobin levels fell from 11 to 7.0 g/100 ml. Below 7.0 g/100 ml, platelet counts may have declined slightly. Line represents the average platelet count fitted by eye. Open circles represent animals on a low-iron diet. Closed circles represent normal control animals.

Table 3. Platelet Production and Blood Counts in Iron Deficiency Anemia Following Iron Injection

Time After Iron (hr)		Hemoglobin (g/100 ml)	Reticulocytes (%)	Platelet Counts ($\times 10^6$)	^{35}S-Radiosulfate (cpm/10^9 Platelets)
0	Control	14.1 ± 0.3	2.2 ± 0.4	1.04 ± 0.11	295 ± 49
	Iron deficient	6.3 ± 1.3	13.0 ± 2.0	2.10 ± 0.35	928 ± 91
24	Control	13.8 ± 0.7	1.6 ± 0.5	0.95 ± 0.21	130 ± 20
	Iron deficient	7.9 ± 0.6	15.0 ± 0.5	1.97 ± 0.23	336 ± 27
48	Control	14.9 ± 0.6	2.0 ± 1.1	1.15 ± 0.72	371 ± 47
	Iron deficient	7.2 ± 0.3	14.0 ± 2.2	1.61 ± 0.28	797 ± 52
72	Control	14.5 ± 0.3	2.2 ± 0.2	1.21 ± 0.20	296 ± 30
	Iron deficient	9.5 ± 0.3	26.1 ± 1.9	1.31 ± 0.20	310 ± 32

Each value represents the mean of at least four rats ± 1 SD.

Platelet-Hematocrit Relationship

Abildgaard and Simone[7] suggested that thrombocytosis in iron deficiency anemia is a hemostatic compensatory mechanism for low hematocrit. Several experiments were performed to test this hypothesis. Platelet production was measured in animals with iron deficiency anemia after transfusion with either iron-deficient red cells or normal red cells sufficient to raise the hematocrit to normal. As seen in Table 4, radiosulfate incorporation in animals with iron deficiency anemia was 3.1 times control values, but after transfusion with either iron-deficient or normal red cells, incorporation was 2.1–2.5 times the normal controls. There was no significant difference between the groups transfused either with normal or iron-deficient red cells. Restoration of the hematocrit to nearly normal levels was associated with a fall in platelet production to levels intermediate between that seen in control and iron-deficient rats. The number of bone marrow megakaryocytes was the same in all but the untreated iron-deficient group, which had consistently fewer cells.

To explore further the platelet-hematocrit relationship, platelet production was measured in anemia without iron deficiency. Normal animals were given phenylhydrazine (1.5 ml of 1% solution daily for 3 days). Radiosulfate was injected 24 hr after the third dose of phenylhydrazine, and animals were sacrificed 24 hr later. By the day of radiosulfate injection, hematocrits fell to 26%–29.5%, and reticulocyte counts rose to 35%–43%. However, platelet counts remained in the normal range (1.12–1.27 × 10^6/cu mm), and platelet production averaged 1.2 times control values, not a significant increase. To study the effect of hematocrit elevation on platelet production, normal animals were made polycythemic by repeated transfusions of normal packed red cells, which after 8 days raised hematocrits to 70%–77% in five animals with a mean of 74% (control 45% in four animals). Platelet counts ranged from 0.70 to 1.19 × 10^6/cu mm with a mean of 0.90 × 10^6/cu mm (control 1.04), and radiosulfate incorporation was slightly below controls (0.8) but was not significantly different.

Table 4. Platelet-Hematocrit Relationship in Iron Deficiency Anemia in Rats

	Hb (g/100 ml)	Hematocrit (%)	Reticulocytes (%)	Platelets (X 10^6/cu mm)	S^{35}-Platelet Incorporation (cmp/10^9 Platelets)
Control	14.1 (±1.6)	43.8 (±0.6)	2.7 (±0.5)	1.021 (±0.091)	295 (±49)
Iron deficiency anemia	7.1 (±0.2)	24.4 (±0.7)	9.4 (±0.5)	2.089 (±0.305)	928 (±91)
Iron deficiency anemia transfused with normal red cells	12.5 (±0.6)	41.1 (±2.8)	6.8 (±1.4)	1.349 (±0.257)	632 (±75)
Iron deficiency anemia transfused with iron-deficient red cells	12.4 (±0.4)	41.2 (±1.2)	6.1 (±0.5)	1.372 (±0.235)	735 (±61)

Each value represents mean of four animals (±SD).

While these studies revealed a significant platelet-hematocrit relationship only in iron deficiency anemia, it is possible that the same relationship might be seen in hemolytic anemia or polycythemia maintained for a longer period of time.

Platelet Survival in Iron Deficiency Anemia

Maximum radiosulfate labeling occurred on the sixth day in control and in iron-deficient animals, and there was no difference in calculated platelet survival, 4.5 days in both groups. These results agree with the findings of Odell and Anderson[6] in normal rats.

DISCUSSION

In iron deficiency anemia in man, altered platelet levels have been reported. Platelet counts may be increased,[1,2] decreased,[1,3] or normal and may change further if complicated by active blood loss or hypersplenism.[8] In our studies of iron-deficient rats, platelet counts were normal or increased. Thrombocytopenia was not observed, but this does not exclude the possible association of thrombocytopenia with severe iron deficiency anemia, particularly if there is a superimposed deficiency of vitamin B_{12},[9] folic acid,[10] or ascorbic acid.[11] Although our data show a rise in platelet count with progressive anemia, platelet counts level off and, in fact, fall toward normal at hemoglobin levels below 7.0 g/100 ml. One may explain the partial reversal of thrombocytosis by the production of larger platelets with no change in total mass or by an increase in plasma volume due to severe anemia.

From our data, it is clear that both platelet production and platelet counts are increased in iron deficiency anemia and that both correlate inversely with the level of hemoglobin. The mean increase in both platelet count and radiosulfate incorporation was two to three times normal. However, in some iron-deficient animals, platelet counts were within normal limits, while radiosulfate incorporation was elevated significantly. This may be explained by a greater sensitivity of radiosulfate incorporation to net changes in platelet production. In iron-deficient rats, platelet production and platelet counts fell to normal within 72 hr following injection of iron. This response correlates well with observations in man,[1,2] although the recovery pattern was faster in rats.

The absolute radioactivity differed in consecutive controls, particularly in the first experiment in Table 1. The reason for this variation is not known. The variation in specific activity of isotope was not sufficient to account for this difference. It is possible that differences in the body pools of sulfate and other ions were responsible for the changes in absolute radioactivity from study to study. Since most control values were similar and the ratio of experimental to controls was reproducible, the conclusions could be drawn with reasonable security.

There is little available information on the relationship of iron deficiency anemia and platelet production. Harker and Finch[13] studied platelet production in two adults with iron deficiency anemia and reactive thrombocytosis. They found an increased number of megakaryocytes in the bone marrow. These results differ from our observations in which the marrow of rats with moderate

anemia contained normal numbers and the marrow ot the rats with severe anemia contained decreased numbers of megakaryocytes. These results were confirmed by subsequent studies.[12]

Another viewpoint may be obtained by examining the effect on megakaryocytopoiesis in rats of transfusion-induced acute thrombocytosis. Ebbe et al.[14] showed that transfusion thrombocytosis did not alter either the rate of megakaryocyte maturation or the influx of precursor cells into the megakaryocyte compartment, although changes in megakaryocyte size were later demonstrated.[15] Odell et al.[16] and Harker[17] observed that transfusion thrombocytosis resulted in fewer marrow megakaryocytes and a subsequent decrease in platelet count. Transfusion thrombocytosis differs in at least one major aspect from thrombocytosis in iron deficiency anemia. In the latter it develops and progresses over a relatively long period of time and persists. It is possible that a feedback regulatory mechanism would also differ.

The inverse relationship between the hematocrit and platelet count in iron deficiency anemia is in agreement with the observations of Abildgaard and Simone.[7] In animals with iron deficiency anemia, hematocrit correction with either iron-deficient or normal red cells resulted in a fall of the platelet count to normal. However, reducing the hematocrit with phenylhydrazine for 3 days or raising the hematocrit by hypertransfusion for 8 days caused little change in platelet production and platelet counts. It is possible, however, that the duration of altered hematocrit may be important. For example, one may see thrombocytosis in patients with chronic hemolysis due to sickle cell anemia[18] or thrombocytopenia in children with chronic erythrocythemia due to an intracardiac shunt.[19]

The decrease in marrow megakaryocytes that we observed in rats with severe iron deficiency anemia is difficult to reconcile with what others have observed during increased platelet production. Prolonged platelet survival or extramedullary hematopoiesis would offer explanations, but we found normal platelet survival and no evidence of extramedullary hematopoiesis in the livers and spleens of animals with iron deficiency anemia. One explanation compatible with these data is faster maturation time and/or increased polyploidy of megakaryocytes resulting in increased platelet production. The focal point at which thrombocytosis is effected in iron deficiency anemia may be the pluripotent stem cell. Stimulation of erythropoiesis with a block in maturation might result in a shunt of stem cells into a parallel cell line. The mechanism of increased platelet production in iron deficiency anemia is the subject of another study.[12]

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