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Parabiotic Demonstration of a Humoral Factor Affecting Megakaryocyte Size in Sl/Sl^d Mice (40263)[1]

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Sl/Sl^d mice have a genetically determined hemopoietic micro-environmental abnormality that accounts for a severe macrocytic anemia (1). Initial studies of megakaryocytopoiesis showed that they have a normal number and mass of circulating platelets, a half-normal number of marrow megakaryocytes, and megakaryocytes that are larger than normal (2). The size of megakaryocytes in Sl/Sl^d mice responds to perturbations of the platelet count, notably showing a substantial reduction in response to transfusion-induced thrombocytosis (3). Therefore, macromegakaryocytosis in Sl/Sl^d mice is not irreversibly determined by a defect of the fixed tissue cells of the marrow. The results of cross-implantation of bone marrow fragments between Sl/Sl^d mice and their normal littermates implicate a humoral, rather than stromal, factor in the induction of macromegakaryocytosis in Sl/Sl^d mice (4). The present studies utilized parabiosis to look for further evidence of stimulation of megakaryocyte growth by humoral factors in Sl/Sl^d mice and to determine if this effect would be nullified by cross-circulation with a normal mouse.

Materials and methods. Mice. Sl/Sl^d female mice from the WCB6F$_1$ stock and their congenic littermates were purchased from Jackson Laboratories. Experiments were started when the mice were 10–12 weeks of age. All blood samples were obtained by cardiac puncture under ether anesthesia, and tissues were obtained after killing the mice by cervical dislocation. Data collected from non-manipulated Sl/Sl^d and +/+ mice over a 6-year period (1971–1977) were pooled to provide "Historical Controls." The probability that differences between mean values for different groups was due to chance alone was determined with Student's t test.

Parabiosis. Parabiosis was done between pairs of +/+ mice and between +/+ and Sl/Sl^d mice under pentobarbital hypnosis. The mice were attached side-by-side after a longitudinal incision was made in the skin of each. Scapulae and femurs of the parabionts were fastened together with No. 0000 surgical steel wire. The skin was joined with No. 0000 silk and skin clips. To separate parabionts, clips, sutures, and wires were removed, and the wounds were closed with silk and skin clips. The animals were either killed for testing or separated 4 weeks after initiation of parabiosis. Separated mice were tested 4 weeks after separation.

Cross-circulation studies. Red cells from congenic +/+ or heterozygous mice were labeled *in vitro* with ^{51}Cr-sodium chromate, washed, and reconstituted with saline to an hematocrit of about 40%. One parabiont was injected intravenously with 0.5 ml of the labeled cells. Radioactivity in washed red cells from both parabionts was measured 1.5–2 hr later.

Mouse blood platelets were labeled with ^{75}Se-selenomethionine *in vitro*. Each CF$_1$ donor was injected with 2 μCi, i.p., 2 days before its blood was collected. Platelets were separated and concentrated by differential centrifugation from blood anticoagulated with ACD. One parabiont of each of four pairs was injected intravenously with 0.3 ml of platelet concentrate containing 1.7×10^9 platelets. Two hours later, radioactivity was measured in the washed platelets from the blood of all parabionts. Previous studies demonstrated the capability of CF$_1$ platelets to recirculate after transfusion into Sl/Sl^d or +/+ mice (3). Preliminary tests utilized CF$_1$ donors and recipients to study this technique for labeling platelets for transfusion. About

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60% of the transfused platelets were recovered 1 hr after transfusion in five normal recipients, and the rate of fall of platelet-bound radioactivity in nine additional recipients indicated a platelet survival of 4–5 days.

Radiation experiments. Six +/+ mice were irradiated with 850 r gamma ray (^{60}Co). Within 3 hr, each was injected intravenously with 4.9×10^6 (0.27 ml) Sl/Sl^d femoral bone marrow cells suspended in TC 199 (pH 7.3). The recipients were killed for blood counts and megakaryocyte size measurements 46 days later.

One or both tibiae of Sl/Sl^d and +/+ mice were irradiated with 950 r gamma ray (^{137}Cs) with the rest of their bodies shielded with lead. Six weeks later, measurements were made of blood counts and megakaryocyte sizes in irradiated tibial and unirradiated humeral marrow.

Blood, bone marrow and spleen counts. Platelets were counted by phase microscopy (5). Techniques for other measurements are described in detail elsewhere (6). Sizes of mature, stage III megakaryocytes were measured from photographs taken from femoral marrow smears; results were expressed as planimeter units. The average size was determined for 32–37 megakaryocytes for each mouse; this average was used for further analysis. Suspensions of tibial marrow cells were used for counts of megakaryocytes by microscopy and total cells by Coulter counter. Megakaryocytes were counted in sub-serial histologic sections, 12 microns thick, of spleens, and the average number per section was calculated for each mouse.

Results. Prompt cross-circulation of red cells and platelets between parabionts was found. Five Sl/Sl^d – +/+ pairs showed less than 5% difference in the level of circulating radioactivity (cpm/ml red cells) after injection of one partner with red cells labeled with ^{51}Cr. There was less than 15% difference in 3 of 4 +/+ pairs, and one pair showed a difference of 46%. After injection of ^{75}Se-labeled platelets into the Sl/Sl^d partner of 3 Sl/Sl^d – +/+ pairs, platelet-bound radioactivity (expressed as cpm in the platelets in 1 ml of blood) was consistently greater in the uninjected +/+ partner. The Sl/Sl^d mice had 58–83% as much radioactivity as the +/+ partners. In one +/+ pair, platelets of the uninjected partner had 74% as much radioactivity as those of the injected mouse. Thus, in all of the mice tested, substantial effective cross-circulation occurred.

Megakaryocytes of +/+ mice paired with Sl/Sl^d (Fig. 1, 2nd bar) were significantly larger than those of +/+ pairs (Fig. 1, 1st bar) ($P < 0.001$). However, the megakaryocytes of +/+ pairs were larger at the end of parabiosis (Fig. 1, 1st bar) than they were 4 weeks after separation (Fig. 1, 3rd bar) ($P < 0.001$) when their size was comparable to that of the historical controls.

In mixed parabionts (Sl/Sl^d – +/+), megakaryocytes of the Sl/Sl^d partner (Fig. 1, 5th bar) were larger than those of the +/+ parabiont (Fig. 1, 2nd bar) ($P < 0.05$). By comparison with historical controls, it is of note that the size of megakaryocytes in Sl/Sl^d mice was not decreased by cross-circulation with a +/+ partner. Because of poor survival after

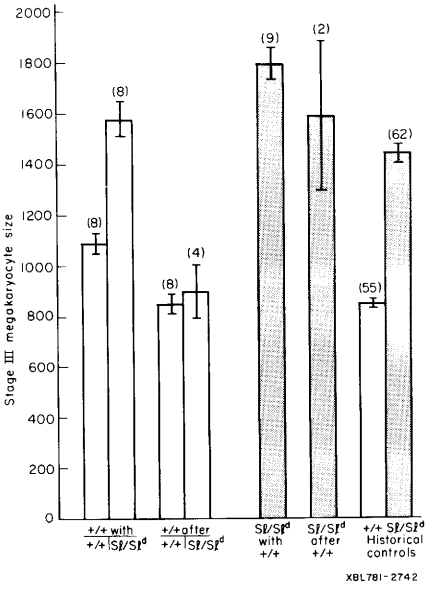

FIG. 1. Megakaryocyte sizes, expressed in arbitrary units; each bar indicates the average ± SEM for the number of mice shown in parentheses. Open bars represent +/+ mice; shaded bars represent Sl/Sl^d mice. From left to right: the first bar represents +/+ parabiosed to +/+ for 4 weeks; the second +/+ parabiosed to Sl/Sl^d; the third and fourth bars show +/+ 4 weeks after separation from +/+ or Sl/Sl^d, respectively; the fifth bar shows Sl/Sl^d parabiosed to +/+; the sixth bar shows Sl/Sl^d 4 weeks after separation from +/+; the seventh and eighth bars show +/+ and Sl/Sl^d historical controls, accumulated during the years 1971–1977.

TABLE I. BLOOD COUNTS AND MEGAKARYOCYTE SIZES OF IRRADIATED MICE.

Group	n	Platelets/mm^3 ($\times 10^{-6}$)	Hct (%)	MCV (μ^3)	Retics (%)	Megakaryocyte size (units)
Irradiated +/+ recipients of Sl/Sl^d marrow cells	6	1.145 ± 0.058*	41.9 ± 0.5	43.6 ± 1.2	2.8 ± 0.2	1143.6 ± 39.3
+/+ partially irradiated	7	1.384 ± 0.079	45.7 ± 0.6	—	2.0 ± 0.3	930.4 ± 42.0 (irradiated) 951.0 ± 48.6 (nonirradiated)
Sl/Sl^d partially irradiated	7	1.379 ± 0.057	30.0 ± 1.4	—	5.9 ± 1.1	1625.1 ± 71.8 (irradiated) 1714.0 ± 102.5 (nonirradiated)

* Mean ± SEM.

TABLE II. MARROW AND SPLEEN CELLS OF PARABIOTIC MICE.[a]

Group	n	Tibial marrow		Spleen
		Nucleated Cells ($\times 10^{-6}$)	Megakaryocytes ($\times 10^{-3}$)	Megakaryocytes per section
+/+				
With +/+	8	17.7 ± 0.9[b] (1)[c]	10.2 ± 1.9	91.2 ± 12.3[c] (3) (4)
With Sl/Sl^d	8	13.0 ± 0.8 (1) (2)	6.8 ± 1.6	242.8 ± 30.7 (3) (5)
After +/+	8	16.7 ± 0.4	15.4 ± 1.5	201.8 ± 18.8 (4)
After Sl/Sl^d	4	16.8 ± 1.0	15.6 ± 1.4	344.8 ± 133.5
Historical controls	19–37	14.4 ± 0.4	11.0 ± 0.6	110.2 ± 5.8
Sl/Sl^d				
With +/+	9	8.7 ± 0.6 (2)	4.0 ± 0.7	14.4 ± 3.0 (5)
After +/+	2	8.8 ± 0.8	6.6 ± 1.4	49.4 ± 12.0
Historical controls	24–42	8.0 ± 0.2	5.0 ± 0.3	88.3 ± 13.2

[a] Measurements made in +/+ parabions and +/+ historical controls are presented in the first five lines; Sl/Sl^d parabions and historical controls are shown in the last three lines. "With" denotes values obtained at the end of 4 weeks of parabiosis to the indicated partner. "After" denotes values obtained after 4 weeks of parabiosis and 4 additional weeks of separation from the indicated partner.

[b] Mean ± SEM.

[c] Statistical analyses refer to results indicated by paired numbers (1) $P < 0.01$. (2) – (5) $P < 0.001$.

termination of parabiosis, recovery of sufficient numbers of Sl/Sl^d partners was not observed for statistically valid comparisons.

Megakaryocyte size distribution curves were unimodal in all mice, thus not suggesting the growth of two intrinsically different populations of cells (Sl/Sl^d and +/+) in the parabionts. To further test the possibility that macromegakaryocytosis in +/+ partners of Sl/Sl^d mice may have been due to transfer and growth of stem cells, marrow cells from Sl/Sl^d mice were transplanted into lethally irradiated +/+ hosts. Platelet counts were somewhat lower than in other groups of +/+ mice, and red cells were normocytic 46 days later (Table I, line 1). Megakaryocyte size was the same as that in +/+ parabiotic pairs (Fig. 1, 1st bar), and it was significantly less than that in the +/+ partners of Sl/Sl^d mice (Fig. 1, 2nd bar) ($P < 0.001$).

To determine if the slight macromegakaryocytosis in irradiated +/+ recipients of marrow cells from Sl/Sl^d mice was produced by radiation damage to bone marrow stroma, the results were compared with findings in partially irradiated Sl/Sl^d and +/+ mice (Table I, lines 2 and 3). Blood counts were identical 6 weeks after irradiation of one or both tibiae, so the results were pooled. Megakaryocytes growing in irradiated tibial marrow or in unirradiated humeral marrow were of identical size in both genotypes.

Parabiosis with Sl/Sl^d partners produced an increase in splenic megakaryocytes and modest tibial hypocellularity in +/+ mice (Table II, line 2) in comparison to the findings in +/+ – +/+ parabionts (Table II, line 1). After termination of parabiosis, tibial cellularity recovered, but splenic megakaryocytosis did not (Table II, line 4). The Sl/Sl^d partners (Table II, line 6) showed their usual level of marrow cells, but splenic megakary-

cytes were substantially lower than those of historical controls. After separation of +/+ pairs, there was a delayed increase in splenic megakaryocytes (Table II, line 3) that was not apparent at the end of 4 weeks of parabiosis (Table II, line 1).

When compared to +/+ pairs (Table III, line 1), partners of Sl/Sl^d mice (Table III, line 2) were slightly anemic ($P < 0.01$), and they showed evidence of stimulated erythropoiesis with reticulocytosis ($P < 0.05$) and macrocytosis ($P < 0.001$). While attached to a +/+ partner, the anemia and macrocytosis of Sl/Sl^d mice was partially corrected (Table III, line 6), and there were no differences in blood counts between the two partners (Table III, lines 2 and 6). Parabiosis itself induced modest thrombocytosis; platelet counts of +/+ pairs (Table III, line 1) were greater than they were 4 weeks after separation (Table III, line 3) ($P < 0.05$). After parabiosis with Sl/Sl^d mice, the platelet counts of the +/+ partners did not go down (Table III, line 4).

Discussion. The present results support the hypothesis that Sl/Sl^d mice produce a humoral factor that causes megakaryocytes to be larger than normal. Megakaryocytes are known to become macrocytic in response to acute thrombocytopenia (7, 8) suggesting that macrocytosis may be a cellular manifestation of the action of a humoral thrombopoietin. It has been proposed that Sl/Sl^d platelets may be intrinsically lacking in a property that is monitored by the homeostatic feed-back mechanism responsible for regulating production of a thrombopoietin, (4) thus leading to macromegakaryocytosis by inappropropriate thrombopoietin secretion. Thrombopoietin assays have not been reported for Sl/Sl^d mice. However, the degree of megakaryocytic macrocytosis in Sl/Sl^d mice has been reduced by transfusions of sufficient numbers of normal platelets to produce thrombocytosis, and Sl/Sl^d mice appeared to be unusually susceptible to this feed-back inhibition by transfusion-induced thrombocytosis (3). Substantial cross-circulation of platelets and red cells between parabiotic pairs was shown in the present experiments indicating that each Sl/Sl^d and +/+ partner probably had platelets of both Sl/Sl^d and +/+ origins in its circulation. Normalization of hematocrit and mean red cell volume in Sl/Sl^d partners of +/+ mice indicated that erythropoiesis oc-

TABLE III. BLOOD COUNTS OF PARABIOTIC MICE.[a]

Group	Platelets/mm^3 ($\times 10^6$)	Hct (%)	MCV (μ^3)	Retics (%)
+/+				
With +/+	1.732 ± 0.077* (1) (10)**	49.8 ± 1.3 (2) (10)	45.0 ± 0.7 (3) (8)	2.6 ± 0.2 (4) (8)
With Sl/Sl^d	1.712 ± 0.213 (8)	41.9 ± 2.0 (2) (8)	50.8 ± 0.6 (3) (5)	5.1 ± 1.0 (4) (5)
After +/+	1.468 ± 0.065 (1) (8)	46.1 ± 1.0 (8)	44.4 ± 0.7 (8)	1.6 ± 0.2 (8)
After Sl/Sl^d	1.752 ± 0.174 (4)	46.0 ± 0.4 (4)	45.4 ± 1.8 (4)	1.6 ± 0.5 (4)
Historical controls	1.330 ± 0.024 (74)	45.6 ± 0.5 (70)	45.4 ± 0.5 (27)	1.2 ± 0.2 (16)
Sl/Sl^d				
With +/+	1.559 ± 0.209 (9)	42.1 ± 2.3 (9)	49.7 ± 1.3 (6)	4.9 ± 0.6 (6)
After +/+	1.265 ± 0.080 (2)	28.5 ± 0.5 (2)	65.4 ± 5.0 (2)	5.2 ± 0.3 (2)
Historical controls	1.546 ± 0.046 (79)	27.8 ± 0.6 (69)	74.8 ± 1.8 (30)	4.1 ± 0.7 (16)

[a] Measurements made in +/+ parabions and +/+ historical controls are presented in the first five lines; Sl/Sl^d parabions and historical controls are shown in the last three lines. "With" denotes values obtained at the end of 4 weeks of parabiosis to the indicated partners. "After" denotes values obtained after 4 weeks of parabiosis and 4 additional weeks of separation from the indicated partner. Statistical analyses refer to results indicated by paired numbers. (1) $P < 0.05$. (2) $P < 0.01$. (3) $P < 0.001$. (4) $P < 0.05$.

* Mean ± SEM.

** Number of mice.

curred predominantly in the +/+ partner, but platelet production could not be identified with one of the partners more than the other. Dilution of putative abnormal Sl/Sl^d platelets by normal +/+ platelets should have abrogated a feed-back stimulation that was due to the abnormal platelets. The finding of unmodified macromegakaryocytosis in Sl/Sl^d parabionts and almost equal macrocytosis in their +/+ partners suggests that the Sl/Sl^d mice autonomously generated a thrombopoietic substance, the production of which was not modified by the presence of moderate numbers of normal platelets. That such a thrombopoietic factor may be generated as a mechanism to compensate for intrinsically reduced numbers of megakaryocytes is worthy of consideration and further experimentation.

It has been proposed that erythropoietin (EP) may stimulate thrombocytopoiesis when erythropoiesis is blocked (9, 10) and that this mechanism may explain megakaryocytic abnormalities in non-manipulated Sl/Sl^d mice (2). Hematocritis were nearly normal in Sl/Sl^d − +/+ parabionts, so EP should have been substantially reduced in Sl/Sl^d partners. Persistence of macromegakaryocytes suggests that they were not related to the anemia and high EP levels present in Sl/Sl^d mice before parabiosis (1).

After irradiation and transplantation with Sl/Sl^d stem cells, +/+ mice showed modest degrees of thrombocytopenia and macromegakaryocytosis. Megakaryocytes did not develop the excessive size associated with parabiosis to an Sl/Sl^d partner, thus suggesting that the findings with parabiosis were not due solely to transfer, growth and differentiation of Sl/Sl^d stem cells in the +/+ environment. Repopulation of the irradiated +/+ hosts by the transplanted Sl/Sl^d stem cells rather than by endogenous stem cells was presumed and was not documented by the use of cell markers. The modest macromegakaryocytosis in transplanted +/+ hosts could not be attributed to radiation effects on marrow stroma, and the only apparent explanation for it was the mild residual thrombocytopenia.

Megakaryocytopoiesis may be stimulated by "nonspecific" (nonthrombocytopenic) conditions, and previous observations have indicated that nonspecific stimuli produce increased numbers of megakaryocytes that mature more rapidly than normal but not macromegakaryocytosis (7, 11, 12). Modest macrocytosis of megakaryocytes occurred in nonthrombocytopenic parabiotic +/+ mice, in which the total stimulus was considered to be of the nonspecific type, thus suggesting the macrocytosis may, in fact, result from potent nonspecific stimulation. The modest thrombocytosis and increases or decreases in splenic megakaryocytes or marrow cells observed during or after parabiosis may have occurred in response to profound reactions to the extensive surgical procedures and subsequent healing. With the present data these changes in numbers of cells can not be attributed solely to nonspecific or genetically determined influences or to specific combinations. Independent of these inexplicable changes, however, it seems to be clear that Sl/Sl^d mice have a humoral factor that affects megakaryocyte growth.

Summary. Parabiosis was done between Sl/Sl^d mice and their normal +/+ littermates and between pairs of +/+ mice. The purpose was to determine if Sl/Sl^d mice produced a substance that would stimulate megakaryocytopoiesis. It was found that +/+ mice attached to Sl/Sl^d mice showed macromegakaryocytosis suggesting that megakaryocytopoiesis was stimulated. Nonspecific findings in +/+ parabiotic pairs included a lesser degree of megakaryocytic macrocytosis. These findings did not appear to be due to growth of Sl/Sl^d stem cells in the +/+ parabionts. Radiation to Sl/Sl^d or +/+ bone marrow did not change the size of megakaryocytes. The results suggest that Sl/Sl^d mice produce a thrombopoietic substance even though they have a normal complement of circulating platelets.

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