

Reduced Blood and Marrow Neutrophils and Granulocytic Colony-Forming Cells in SI/SI^d Mice¹ (39405)

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The SI/SI^d mouse is anemic (1), has reduced numbers of megakaryocytes but normal levels of blood platelets (2), and its defective hematopoiesis is thought to be due to a defect in the hematopoietic microenvironment. This latter conclusion is based on a series of observations. The animal will not support growth of normal sized spleen colonies on its own spleen (3), but if intact littermate spleens are transplanted in SI/SI^d they will support normal colony growth (4). Transplantation of intact littermate spleens improves the anemia (5) but intravenous injection of spleen or marrow cells does not (3). Marrow or spleen cells from SI/SI^d grow normally when injected into irradiated littermates or syngeneic W/W^v (3). These studies thus suggest that its hematopoietic stem cells are normal, that levels of "poietins" are adequate and by default implicate the poorly defined microenvironment. The reduction of megakaryocytes (1) indicates the defect is not confined to erythropoiesis and suggests that feed-out from the pluripotent stem cell compartment might be reduced. If so, then reduced neutrophils and neutrophil precursors should also be present. In this study we measured blood and marrow neutrophils and neutrophil precursors, number of marrow cells forming granulocytic colonies in semisolid culture, marrow growth in liquid culture, and production of colony-stimulating activity from macrophages.

Materials and methods. All methods used have been reported in detail and will only be summarized.

Mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Mice of var-

ious ages were used, but in each experiment SI/SI^d and the normal littermates (+/+) were matched for age. Blood was obtained by puncturing the lateral orbital sinus with a microhematocrit tube containing heparin. Duplicate total leukocyte counts were done electronically and 200 cell differential counts were done on Wright's stained smears (6). Nucleated cells were washed from both humeri, duplicate counts were done electronically, and results were expressed as the mean value from both humeri (6). The method removes more than 90% of nucleated cells and is reasonably precise in that counts rarely vary by more than 10% between the humeri of the same mouse. An aliquot of the marrow suspension is passed through a millipore filter (pore size 45 μ m), cells on the filter stained for peroxidase and the percentage peroxidase positive cells determined by counting 500 cells.

For semisolid culture, 10⁵ nucleated cells from a femur are added to 1 ml of methyl cellulose, CMRL culture media, horse serum, and colony-stimulating activity from L cells, and the mixture was added to a petri dish (7). Dishes are incubated at 37° in humidified 10% CO₂ and colonies (more than 50 cells) counted after 4 and 7 days. Liquid cultures in test tubes, 12 × 75-mm polyethylene (Falcon plastics), were set up in the same media but without methyl cellulose (8). One culture tube from each mouse was sacrificed at 2, 4, 6, and 8 days of culture and total cells were determined electronically.

Results. Twelve-week-old SI/SI^d mice were compared with littermate controls (+/+) with respect to blood and marrow (Table I). Moderate anemia was present in SI/SI^d with hematocrit values averaging 65% of those of the +/+. Total leukocyte count on the first tube of blood drawn from the orbital sinus did not differ between the two groups. However, the percentage of neutro-

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TABLE I. GRANULOCYTOPOIESIS IN SL/SL^d MICE AND THEIR LITTERMATE CONTROLS

	Mice*		P values
	Sl/Sl ^d (Mean ± SE)	+/+	
Mouse weight (g)	21 ± 1	22 ± 1	NS
Blood			
Hematocrit (%)	31 ± 1	48 ± 1	<0.01
Leukocytes (thousands/mm ³)	4.1 ± 0.2	4.3 ± 0.2	NS
Neutrophils (thousands/mm ³)	0.8 ± 0.1	1.4 ± 0.2	<0.05
Marrow (humerus)			
Total nucleated cells (millions)	5.7 ± 0.4	9.6 ± 0.4	<0.01
Peroxidase positive (%)	19 ± 2	35 ± 4	<0.05
Total peroxidase positive	1.2 ± 0.1	3.5 ± 0.5	<0.01
Colonies/10 ⁵ nucleated cells	105 ± 8	114 ± 12	NS
Total colony-forming cells/humerus (millions)	0.06 ± 0.01	0.12 ± 0.02	<0.01

* Five mice per group. ** From Student's *t* test; NS indicates nonsignificant or *P* of greater than 0.05.

phils was only 19% in Sl/Sl^d as compared to 32% in +/+, so the total neutrophil concentration in Sl/Sl^d was reduced to 57% of that in +/+ (Table I). The total number of lymphocytes per cubic millimeter was slightly higher in Sl/Sl^d than in +/+, but the difference was not statistically significant. Leukocyte counts and neutrophil concentration were also determined on the seventh tube of blood drawn from the orbital sinus. Total counts were significantly less in Sl/Sl^d than in +/+ (1900 ± 100 vs 2600 ± 200) and there was an even greater reduction in neutrophils than in the first tube (Sl/Sl^d reduced to 43% of +/+, as compared to 57% in first tube counts).

Total nucleated cells per humerus were reduced to 59% of control in Sl/Sl^d (Table I). The percentage of peroxidase positive cells, which correlates well with the percentage of neutrophils and neutrophil precursors counted in Wright's stained smears (6), was also reduced to 54% of controls. Consequently, a profound reduction (34% of control) was present in total peroxidase positive cells per humerus (Table I). The proportion of marrow cells forming colonies in semi-solid media was quite similar in Sl/Sl^d and +/+. However, as a result of the decrease in total cells per humerus, the total number of colony-forming cells was decreased significantly in Sl/Sl^d (Table I).

All studies were repeated in 8-week-old Sl/Sl^d and all of the above differences between Sl/Sl^d and littermates were confirmed (results not shown).

Colony size was estimated on Day 4 and

Day 7 with the observer being unaware of whether the plate contained marrow from Sl/Sl^d or from +/+. Colonies were estimated as containing 50-100, 100-500, 500-1000, or greater than 1000 cells. Clusters (20-50 cells) were also scored. No significant difference in colony size was discerned between Sl/Sl^d and +/+. For example, 32% of all colonies and clusters were estimated to contain more than 100 cells in Sl/Sl^d as compared to 31% in +/+

In order to further study growth rate of cells, which colony size presumably reflects, studies were done in liquid culture so that accurate cell counts could be done. One-hundred-fifty-thousand nucleated marrow cells from each of 4 Sl/Sl^d and +/+ were cultured. At 2, 4, and 6 days nucleated cells were 148,000, 182,000, and 235,000 in +/+ and 152,000, 180,000, and 232,000 in Sl/Sl^d cultures. In a repeat study, carried to 8 days, again no significant difference was observed between the number of cells in cultures from +/+ or Sl/Sl^d.

Colonies were removed from plates with a Pasteur pipet and smeared on glass coverslips and stained. Smears were also made from liquid cultures. At 4 days most cells appeared to be neutrophil precursors and by 7-8 days almost all appeared to be macrophages. There was no evident difference in morphologic appearance of cells between cultures of Sl/Sl^d and +/+ marrow.

Peritoneal macrophages of Sl/Sl^d were as efficient as those from +/+ when used as a source of colony-stimulating activity (Table II). Addition of peritoneal cells in excess of

the number needed to produce maximal colony stimulation failed to yield any suggestion of production of an inhibitor by the Sl/Sl^d (Table II). The marrow used in the second experiment shown in Table II was from 32-week-old mice and unlike the previously noted studies with 8- and 12-week-old animals there was a significant ($P < 0.01$) reduction in the concentration of colony forming cells in Sl/Sl^d marrow whether stimulated with +/+ or Sl/Sl^d feeder layers or with conditioned media from L cells.

Discussion. Reduced numbers of neutrophils are present in blood and marrow of Sl/Sl^d as compared to +/+ littermate controls. This is not unexpected since, in addition to anemia, the number of megakaryocytes is reduced in this mouse (2). Thus, three major cell lines are affected by the defect. This suggests that the primary problem may reside at the level of the pluripotent stem cell. If so, the defect could reflect a reduced stem cell compartment size (reduced rate of self-replication) or defective induction of differentiation from the compartment. As discussed in the introduction, the Sl/Sl^d defect appears to reside in the hematopoietic microenvironment rather than being an intrinsic defect of the stem cell. The W/W^v mouse, which does have defective stem cells (10), also has reduced marrow neutrophils

(11) and megakaryocytes (11, 12) in addition to being anemic.

The hematopoietic microenvironment is poorly understood (13). It is not clear whether the marrow (and to a lesser degree the spleen) of mice contains some "positive" factor necessary for hematopoietic growth which is missing in other organs, whether the physical structure of the marrow is the key factor or whether other organs contain a "negative" environment.

Hormonal deficit(s) probably cannot be implicated since apparently normal hematopoiesis takes place in +/+ marrow or spleen implanted in Sl/Sl^d (5, 14). Erythropoietin levels appear to be at least appropriate to the degree of anemia (15). Production of colony-stimulating activity from Sl/Sl^d peritoneal cells was normal in our study as it was from bone marrow cells in preliminary reports from other laboratories (16, 17).

The cell-forming colonies of neutrophils and monocytes in semisolid culture is thought to be a more differentiated cell than the pluripotent stem cell. Whether it is a true stem cell, i.e., capable of extensive self-replication, remains to be proven unequivocally. This cell is often referred to as a CFU_C (colony-forming unit in culture) while the pluripotent cell is termed a CFU_S (colony-forming unit in spleen of irradiated

TABLE II. ABILITY OF MACROPHAGES FROM +/+ AND Sl/Sl^d TO STIMULATE COLONY FORMATION FROM +/+ AND Sl/Sl^d MARROW.

Experiment number	Source of colony-stimulating activity	Target marrow (10 ⁶ marrow cells)	
		+/+	Sl/Sl ^d
		Number colonies/plate (Mean ± SE)	
	Conditioned media from +/+ *	39 ± 4	—
	Conditioned media from Sl/Sl ^d	32 ± 3	—
2	Feeder layer of peritoneal cells from +/+ ** (5 × 10 ⁵ cells)	95 ± 4	68 ± 3
	Feeder layer of peritoneal cells from Sl/Sl ^d (5 × 10 ⁵ cells)	92 ± 6	72 ± 6
	Feeder layer of peritoneal cells from +/+ (10 ⁶ cells)	96 ± 7	71 ± 2
	Feeder layer of peritoneal cells from Sl/Sl ^d (10 ⁶ cells)	91 ± 5	67 ± 10
	L-Cell-conditioned media ***	104 ± 4	69 ± 2

* Peritoneal macrophages were collected by placing a 1-cm² of cellulose acetate in the peritoneum for 1 week. Such membranes are covered with a uniform monolayer (9). Macrophage monolayers were incubated for 1 week in CMRL tissue culture media and the supernate harvested and used as a CSA source. Four membranes from four mice in each group were studied.

** The peritoneal cavity of freshly killed, untreated mice was lavaged with CMRL media. Approximately 60% of the harvested cells appeared to be macrophages and the remainder appeared lymphoid. Peritoneal cells from three mice in each group were studied.

*** Used at maximal stimulation level.

mice). The best evidence that the CFU_C differs from the CFU_S is found in studies of the W/W^v mouse. CFU_S is clearly defective, but W/W^v marrow contains a normal concentration of CFU_C (18). The total number of CFU_C is reduced because of the total number of nucleated cells is reduced (10), but they grow colonies of normal size *in vitro* (18; and unpublished studies from our laboratory).

The concentration of CFU_C in marrow was normal in SI/SI^d aged 8 and 12 weeks, but significantly less than that of controls in one group of 32-week-old mice. However, since the total number of nucleated cells in marrow was reduced in the SI/SI^d, total CFU_C per humerus were reduced in all age groups studied, in basic agreement with preliminary reports from other laboratories (16, 17). McCulloch *et al.* (18) found the concentration of CFU_C in spleens of SI/SI^d to be reduced in three of four studies. Sutherland, Till, and McCulloch (19) studied the rate of repopulation of the marrow and spleen of irradiated SI/SI^d recipients of +/+ marrow as compared to +/+ recipients. The rate of repopulation of CFU_C was suggestively reduced and peroxidase-positive cells definitely reduced in SI/SI^d marrow and both rates were even more profoundly reduced in spleen. The reduction in CFU_C certainly is not disproportionate to the overall reduction in neutrophils and is compatible with the defect primarily affecting rate of feedout of cells from the CFU_S compartment. The concentration of CFU_S in SI/SI^d marrow is reduced (3, 22) as it is in the spleen (21). McCarthy found that the total number of CFU_S in femur, spleen, and blood was also reduced (22). CFU_S also are decreased in fetal liver of SI/SI^d (23). Therefore, reduced feedout from the compartment could simply be an expression of reduced compartment size or, alternatively, there could be a defect in rate of initiating differentiation.

The normal growth of cells in semisolid or liquid cultures is what would be anticipated in view of apparently normal growth of SI/SI^d cells in irradiated normal littermates (3, 21).

Although megakaryocytes are reduced in number, platelet counts are normal in SI/SI^d (2) and W/W^v (12). The normal platelet

count probably reflects increased platelet production per megakaryocyte (2, 11, 24). The W/W^v usually maintains normal blood neutrophil levels despite a 50% reduction in marrow neutrophils and neutrophil precursors (11). Since the SI/SI^d had a more severe reduction in marrow neutrophils than the W/W^v, it is not surprising that they were neutropenic. SI/SI^d also are more anemic and more radiosensitive than W/W^v (5), so it seems probable the stem cell compartment is more severely compromised by the SI/SI^d defect.

As judged by response to epinephrine, patients with neutropenia have a greater proportion of cells in the marginal blood pool than in the freely circulating pool (25). The mouse has no change in leukocytes after epinephrine injection (unpublished observations), but an assessment of the size of marginal and circulating pools can be obtained from the ratio of neutrophil counts in the first and seventh tube of blood obtained from the orbital sinus (6). The first tube of blood from the orbital sinus as well as the first drop from a tail vein or the first sample from the vena cava have significantly higher leukocyte concentrations than does the aorta of the mouse (6). As repeated tubes are drawn from the orbital sinus, leukocyte concentration declines and by the seventh tube approximates counts from the aorta. The most reasonable explanation seems to be that the marginal as well as the circulating pool is represented in the first tube while the aorta represents only the circulating pool. Thus, the ratio of first to seventh tube counts provides an estimate of the relative size of the marginal and circulating pools. In +/+ this ratio was 60% while in SI/SI^d it was 45%. This suggests that the circulating pool was reduced to a greater degree than the marginal pool in SI/SI^d animals.

Summary. The SI/SI^d mouse has been shown to have anemia and reduced megakaryocytes, but neutrophil levels in blood and marrow have not been studied previously to our knowledge. It proved to be neutropenic with reduced neutrophils and neutrophil precursors in marrow. The concentration of marrow cells forming granulocytic colonies in semisolid media was normal in young mice, but the total number per

humerus was reduced. *In vitro* growth appeared normal in both semisolid and liquid culture, and peritoneal cells produced colony-stimulating activity normally. These results are compatible with previous studies indicating the Sl/Sl^d defect lies in the hematopoietic microenvironment rather than being a primary cellular defect, and suggest feedout from the pluripotent stem cell compartment may be reduced.

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