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Granulocytopoiesis: Studies on Leukocytosis-Inducing and Colony-Stimulating Factors (39635)[1, 2]

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Introduction. Two humoral agents, neutrophilic leukocytosis-inducing factor (LIF)[3] and colony-stimulating factor (CSF)[4] are believed to assist in maintaining normal granulocyte levels in the blood and marrow (1, 2). This concept is based on the cyclic fluctuation seen in the blood neutrophil level of some normal individuals and the presence of a large reservoir of mature neutrophilic cells in the marrow (1-3). According to this concept, when the blood neutrophil level reaches the low normal, LIF is released which accelerates the mobilization of mature neutrophils from the marrow reserve into the blood. In addition, reduction in blood neutrophil level is considered to result in release of CSF which is believed to stimulate proliferation and differentiation of the stem cells, myeloblasts, promyelocytes and myelocytes in the marrow (1, 2, 4-6). This concept is quite attractive and is supported by studies suggesting that LIF is a different moiety from CSF (4, 7).

The purpose of the current studies was to clarify further the relationship between leukocytosis-inducing factor and colony-stimulating factor activities.

Methods. Peritoneal lavages. Four healthy mongrel dogs weighing 15-20 kg were splenectomized in order to exclude margination and demargination of granulocytes during the peritoneal lavage and LIF testing procedures. Two months or more after the surgery and when their hemograms were normal, the dogs were subjected to peritoneal lavages under strict aseptic conditions as described for peritoneal dialysis (8). After 1 ml of blood was obtained for total and differential leukocyte counts and a marrow aspirate was taken for differential counts as described below, 2 g of sterile potato starch in 1000 ml of warm normal saline was infused into the peritoneal cavity. From three of the dogs, a fraction (~200 ml) of the infusate was withdrawn immediately and labeled as control lavage fluid. Eight hours later, blood and marrow samples were again taken for the purposes stated above. The peritoneal fluid was collected into blood collecting bags and was labeled as lavage I. The cavity was washed with 3-4 liter of warm normal saline. At the end of washing, 2 g of potato starch in 1000 ml of warm normal saline was infused. Eight and eighteen hours later, the above procedure was repeated (except that potato starch was not infused after the last lavage), and the lavages were labeled as II and III. Total and differential leukocyte counts were done on the lavages and washes as on the blood samples. The washes were discarded. The lavages were centrifuged at 1500*g* for 15 min to obtain cell-free fluid. All fluids were cultured for aerobic and anaerobic bacteria. The fluids were filtered using 0.45 μm Nalge filters[5] and frozen at −20°C. From these samples, CSF and LIF activities were determined. Before assaying the fluids for CSF and LIF, limulus assay (9) was done on samples from two dogs to detect endotoxin contamination.

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[2] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

[3] LIF refers to neutrophilic leukocytosis-inducing factor activity. It is also known as neutrophil-releasing factor, neutrophilia-inducing factor, and leukocytosis-inducing factor.

[4] CSF refers to the ability of a sample to stimulate proliferation of granulocytic-macrophage colonies from target marrow cells *in vitro* in soft agar medium.

[5] Sybrom Corporation, Rochester, N.Y.

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LIF and CSF are believed to be α-globulins (10, 11). To determine whether a correlation exists between the levels of LIF and CSF with α-globulins, the α-globulins as well as the total protein in the lavage fluids were quantitated by electrophoresis on cellulose acetate strips.

Handling of blood and marrow samples. Total blood leukocyte counts were obtained with the Coulter Model F counter, and differential counts, by counting 200 cells on Wright–Giemsa stained smears. Marrow nucleated cell differential counts were done by scanning 500 cells on Wright–Giemsa stained smears.

Effect of Escherichia coli endotoxin on the blood neutrophils. After three of the eight lavage fluids tested by limulus assay were found to contain endotoxin in concentrations up to 0.3 ng/ml, a study was done to determine whether the amounts of endotoxin present in the lavage fluids injected for LIF assay were responsible for the observed postinjection blood neutrophil changes. *Escherichia coli* endotoxin,[6] at a dose of 28 ng/dog for two dogs and 84 ng/dog for three dogs, was injected iv in 10 ml of normal saline in 1–2 min. Blood samples (1 ml each) were obtained from these dogs immediately before and after the injection and at 1, 2, 4, 6, 12, and 24 hr after the injection. Total and differential leukocyte counts were obtained from these samples as noted above.

LIF in the lavage fluid samples. Approximately 6 weeks after lavage, the lavage fluids were tested for LIF activity in the dog from which they were obtained, with at least 3 weeks allowed to elapse between testing of different fluids. Each lavage fluid was tested as follows. After 1 ml of blood was obtained for total and differential leukocyte counts, a lavage fluid (5 ml/kg body wt) was infused iv in 5 min. At 1, 2, 4, 6, 12, and 24 hr after the injection, blood samples (1 ml each) were obtained for total and differential leukocyte counts. The maximum elevation of the blood neutrophilic band and segmented forms obtained after the injection of a fluid, expressed as the percentage of the immediate preinjection value, was taken as the LIF activity of the fluid.

CSF in the lavage fluids. CSF activity in the lavage fluids was determined *in vitro* by the method of Marsh *et al.* (12). Homologous marrow cells, 2×10^5 per plate and three–five plates per sample, were used as target cells. A group of ≥ 17 cells was defined as a colony.

Lavage fluids from two dogs were dialyzed against distilled water for 48 hr to exclude the presence of inhibitors (13). Both dialyzed and nondialyzed samples from these dogs were tested simultaneously for CSF activity.

Results. Cells removed in lavages and granulocytic cellularity in the blood and marrow during lavages. The numbers of cells removed from the peritoneal lavages of the four dogs are shown in Fig. 1; also shown are the blood and marrow neutrophil changes noted before, during, and for 2 days after lavages. The numbers of total cells and of various types of cells removed were about the same in the three lavages. Total band plus segmented forms and monocytes removed at each lavage ranged from 11.4 to 16.8 $\times 10^9$ and 1.8 to 2.7 $\times 10^9$, respectively.

Blood band forms were 140/mm^3 at basal level and were 860, 640, and 630/mm^3 at lavages I, II, and III, respectively. The segmented forms were 5.5×10^3 at basal level and were 17.0, 13, and 9.5×10^3/mm^3 at lavages I, II, and III, respectively. Both band and segmented forms were significantly ($P < 0.05$) greater at lavages I, II, and III than at the basal level. At 20 and 40 hr postlavage III, band forms were not significantly ($P > 0.1$) different from those at the basal level.

The marrow M:E ratio (Fig. 1) was significantly ($P < 0.05$) higher at lavages II and III and 20 hr later than at the basal level. The subsequent value was not significantly ($P > 0.1$) different from the basal value. Sequentially, the myeloblasts + promyelocytes and the myelocytes increased in the marrow as the M:E ratio increased. These cells were significantly ($P < 0.05$) higher at lavage III than at the basal level, but, at other points of analysis, they were not significantly ($P > 0.05$) higher. Metamyelo-

[6] Worthington Biochemical Corporation, Freehold, N.J.

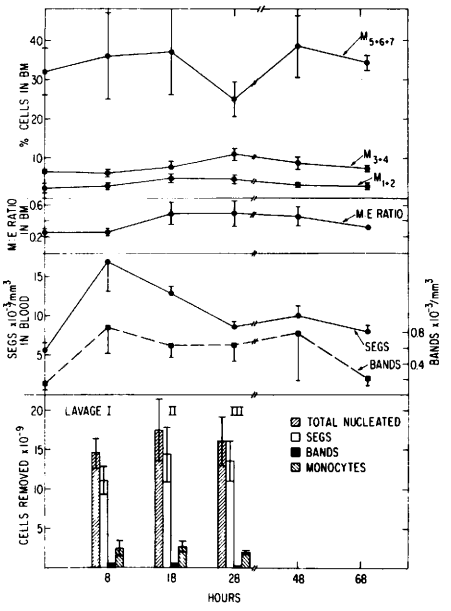

FIG. 1. The results of four studies plotted against time after the first infusion of starch + saline into peritoneal cavities of dogs; mean and SEM are shown. Bottom section: numbers of various types of cells recovered from lavages. Second section from bottom: blood neutrophilic bands and segments. Second section from top: M:E ratio of bone marrow (BM), with M representing myeloblasts + promyelocytes + myelocytes and E, all stages of normoblasts + proerythroblasts + erythroblasts. Top section: marrow differential counts as percent of total nucleated cells. M_1 = myeloblast, M_2 = promyelocyte, M_3 = large myelocyte (> 17.0 μm in diameter), M_4 = small myelocyte (< 16.9 μm in diameter), M_5 = metamyelocyte, M_6 = band form, and M_7 = segmented form.

cytes + band forms + segmented forms decreased to 0.78 times the basal value at lavage III; approximately 20 hr later, they were 1.2 times the basal value, and, in the subsequent sample, they decreased toward the basal value; however, neither the decreased nor the increased value was significantly different from the basal value. Marrow spicules from the aspirates obtained at lavage III and later showed increased granulocytic cellularity.

LIF in the lavage fluids. The pattern of changes in the levels of band and segmented forms in the blood of the dogs following the injection of lavages I, II, or III was similar. Their levels were elevated at 1 or 2 hr (the first determination), and peak levels were reached at 4 to 6 hr after the injection.

LIF activity of the lavage fluids is shown in Fig. 2. In the control lavages it was 34% above the basal level. It was maximum in lavage I, 237%, and showed a slight but insignificant ($P > 0.25$ for lavages I vs II and for I vs III) reduction in lavages II, 199%, and III, 213%. LIF activity in control lavage fluid was different ($P \leq 0.01$) from that in lavage I, II, or III.

Blood neutrophil changes after endotoxin injection. Changes in the blood neutrophilic band and segmented forms of dogs injected with 28 or 84 ng of endotoxin (Table I) were less than those found after control lavage injection. The maximum increase in mean number of band and segmented forms was 21.5% above the basal value after the larger of the two doses of endotoxin was administered.

CSF in lavage fluids. Addition of control lavage fluid to culture plates resulted in production of an average of 11.8 colonies per plate (Fig. 2). The plates with no added stimulus grew ≤10 colonies per plate. The maximum CSF activity, found in lavage I, induced proliferation of 50.8 colonies per plate (430% of the control), compared with 22.2 (188% of the control) and 17.3 (146% of the control) for lavages II and III, respectively. The CSF activity in lavage I was significantly different from that in lavages II ($P > 0.005$ and < 0.01) and III ($P < 0.005$). The activity in lavage II was not significantly

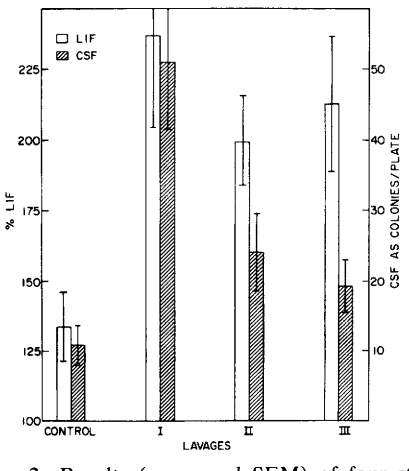

FIG. 2. Results (mean and SEM) of four studies (three for control). LIF activity is maximum elevation of band + segmented forms in the blood after injection of lavage fluid (usually occurring at 4–6 hr), expressed as the percentage of immediate preinjection value. CSF activity is the number of colonies per plate (see text).

TABLE I. NEUTROPHILIC BAND AND SEGMENTED FORMS EXPRESSED AS PERCENTAGE (MEAN AND SEM) OF PREINJECTION VALUE IN THE BLOOD OF DOGS INJECTED WITH *E. coli* ENDOTOXIN.

Endotoxin injected (ng/dog)	Immediate Preinjection	Immediate postinjection	Hours after injection of endotoxin					
			1	2	4	6	12	24
28	100.0	102.0	79.0	69.0	76.0	85.0	82.0	93.0
(Three dogs)	±0.0	±6.5	±3.0	±7.0	±1.4	±9.0	±2.0	±27.9
84	100.0	100.3	96.3	93.3	115.3	112.3	121.5	107.0
(Two dogs)	±0.0	±4.4	±1.4	±7.5	±13.3	±19.3	±14.3	±4.1

different from that in lavage III ($P > 0.2$), and the activity in lavage III was only slightly significantly different from that in the control ($P = 0.05$). There was no correlation ($P > 0.25$) between the LIF and CSF activity levels of lavage fluids II and III, indicating that the CSF and LIF are two distinct entities.

The amount (average of two-dog studies) of $\alpha_1 + \alpha_2$ globulins was 5, 157, 128, and 57 mg%, and the total protein was 40, 824, 465, and 297 mg% in the control lavage and in lavages I, II, and III, respectively. Correlation of α-globulins or total protein with CSF was good ($P < 0.05$), but with LIF it was poor ($P > 0.25$).

The CSF activity was similar in the dialyzed and nondialyzed samples. Results of one study are shown in Table II. These results indicate that there was no dialyzable inhibitor in any of the lavages.

TABLE II. CSF ACTIVITY (MEAN AND SEM OF THREE PLATES) IN DIALYZED AND NONDIALYZED FLUIDS.

Fluid	Lavage number		
	I	II	III
Nondialyzed	128.7 ±7.4	87.7 ±2.9	44.0 ±2.0
Dialyzed	130.0 ±2.8	86.7 ±5.8	50.6 ±1.67

Discussion. Induction of peritoneal inflammation with potato starch and removal of neutrophils from the peritoneal cavities of dogs in this study were associated with rapid mobilization of neutrophilic band and segmented forms from the marrow into the blood. Similar rapid mobilization of cells from the marrow into the blood with lavaging has been shown in rats (14). The lavage procedure was also associated with increased granulocytopoiesis in the marrow as reflected by the increase in the marrow M:E ratio, the percentage of M_{1-4} cells, and the granulocytic cellularity.

The induction of inflammation and lavaging of the peritoneum were also associated with the release of LIF into the peritoneal cavity. The LIF level was probably elevated in the blood of these dogs as well as in the lavage fluids, since plasma samples obtained from rats during peritoneal leukopheresis contained increased LIF levels (15). Injection of the lavages into the dogs from which they were obtained induced elevation in the number of blood neutrophilic band and segmented forms, which indicated rapid mobilization of the neutrophils from the marrow into the blood. Persistence of the elevated LIF activity in all three lavages was not unexpected in view of the sustained demand for the cells in the periphery with continued peritoneal inflammation and lavaging.

The LIF activity demonstrated in the lavages was not due to bacterial contamination since only sterile fluids were tested. Further, the observed LIF activity in the lavages was not due to endotoxin for the following reasons. (i) Blood neutrophil level was already increased in the first blood sample (1–2 hr) obtained after injection of the lavage fluids. It is known that after an endotoxin injection the blood neutrophil level drops for 2–4 hr with a subsequent increase (16). (ii) A maximum of 0.3 ng of endotoxin/ml of lavage fluid was estimated by limulus assay in three out of eight fluids tested before injection into dogs for LIF assay. At this contamination level, the total amount of endotoxin injected into each dog was 30 ng. Injection of 84 ng of E. coli endotoxin/dog in this study (Table I) or as much as 100 ng of *Salmonella abortus equi* endotoxin/dog by others (16) did not perceptibly increase the blood neutrophil count. (iii) In addition, LIF-activity of those lavages with endotoxin (as recognized by limulus test) was not different from the ac-

tivity of those with no detectable contamination.

CSF was also released into the peritoneal cavity in response to the inflammation of the peritoneum. The CSF activity in the lavages was not due to endotoxin contamination, since addition of endotoxin directly to the culture plates did not stimulate colony formation (17). The reasons for the precipitous reduction of CSF activity between lavages I and II are not clear, but the possibilities include rapid catabolism of the produced CSF, exhaustion of CSF stores from the CSF-producing tissue, masking of the CSF by inhibitors, and/or rapid utilization by the target cells. Evidence indicating that the CSF binds to those cells capable of forming granulocytic-macrophage colonies (CFU-C) in *in vitro* cultures (18) supports the last of these possibilities. Masking of the inhibitors was not likely since dialysis of the fluids against water did not reveal the presence of inhibitors; however, the other possibilities cannot be excluded.

The temporal relationship between the reduction in CSF in lavages II and III and increasing M:E ratio and percentage of M_{1-4} cells in the marrow of these dogs suggests that the CSF is probably inducing the M_{1-4} cells to proliferate. CSF has been shown to be essential for the persistence and proliferation of CFU-C in *in vitro* cultures, and, as noted above, it binds to CFU-C (18). The marrow CFU-C are increased in adult mice injected with concentrated human urinary CSF (19). These observations suggest that CSF is influencing granulocytopoiesis *in vivo* by acting on CFU-C and M_{1-4} cells, as has been suggested by other investigators (4, 5). Whether the CSF acts on pluripotent hemopoietic stem cells is not clear.

The nonconcordant levels of LIF and CSF activities demonstrated in lavage fluids II and III indicate that LIF and CSF are two distinct entities. A similar suggestion has been made by others who noted evidence of increased LIF activity and relatively less elevated CSF in the plasma of rats and mice given repeated doses of endotoxin (4, 7). Both LIF and CSF are reported to be α-globulins (10, 11), and CSF has been demonstrated to constitute only a small fraction of the total serum α-globulins (20). In view of these observations it is likely that both LIF and CSF constitute only a small fraction of the total α-globulins in the lavage fluids. Without additional studies on purified preparations, the good correlations observed in lavage fluids between CSF and α-globulins and the lack of such correlation between LIF and α-globulins can be considered only as additional evidence suggesting that the activities of CSF and LIF are mediated by separate moieties.

Summary. Three peritoneal lavages at intervals of approximately 8–10 hr were performed in each of four splenectomized dogs. Induction of peritoneal inflammation and removal of neutrophilic cells from the peritoneal cavity was associated with a rapid mobilization of the neutrophilic band and segmented forms from the marrow into the blood and increased granulocytopoiesis in the marrow. The fluids removed from the peritoneal cavity contained both neutrophilic leukocytosis-inducing factor (LIF) and colony-stimulating factor (CSF) activities. The intensity of blood neutrophilia noted in dogs following the iv injection of fluids was considered to reflect LIF activity. CSF activity was assayed by the capacity of the lavage fluids to stimulate dogs' marrow cells to form granulocytic-macrophage colonies *in vitro* in soft agar medium. The increased LIF activity level was observed in lavages I, II, and III to approximately the same extent. This observation indicated that the production of LIF, having the function of mobilizing mature neutrophils from the marrow into the blood, continued as long as the peripheral demand for the cells existed. The CSF activity was maximum in lavage I and was precipitously reduced in lavages II and III. Following the increase of CSF level in lavages, the marrow M:E ratio, the percentage of myeloblasts + promyelocytes + myelocytes, and the granulocytic cellularity increased, which suggested that the CSF was influencing marrow granulocytopoiesis.

The pattern of fluctuation of the CSF activity in the lavages was different from that of the LIF activity, which indicated that CSF and LIF are two different entities.

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