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Possible Involvement of Leukocytic Endogenous Mediator in Granulopoiesis (39751)

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During the acute phase of a variety of stresses to an animal, the granulocytes release a protein called leukocytic endogenous mediator (LEM), which is indistinguishable from endogenous pyrogen (1–4). LEM has been shown to cause a rapid release of neutrophils from bone marrow to the peripheral blood (5). It will not produce a tolerance upon repeated injection (1, 3, 6) and can, therefore, be used to keep the bone marrow depleted of mature neutrophils.

In the present investigation repeated injections of LEM were administered to rats, and effects upon peripheral blood and bone marrow neutrophils were determined. It also seemed possible that there may be some relationship between LEM and colony-stimulating factor (CSF), since both have been demonstrated in the serum of animals at about the same period of time after injections of endotoxin (7–10). Assays were made, therefore, for CSF in the serum after multiple injections of LEM as well as determinations of the effects of LEM added directly to bone marrow culture plates.

Materials and methods. Animals. Female Holtzman-derived rats from our colony weighing 200–220 g were fed Rockland rat diet and water *ad libitum*. They were maintained at 72°F with 12 hr of light and 12 hr of darkness.

LEM injections. Methods for the preparation of LEM from peritoneal granulocytes have been previously described (1, 2, 5). For the multiple-injection experiments crude LEM was used. It was necessary to concentrate the LEM and add it in a small volume to the bone marrow culture plates. To stabilize the LEM during concentration and storage (11), it was partially purified by the butanol–methanol method (2). The amount of LEM is expressed as the number of granulocytes from which that dose was derived. The multiple injections of 1×10^8 LEM/rat were given at 8-hr intervals at 8 AM, 4 PM, and midnight. Peripheral blood neutrophils were determined 5 hr after the last injection, at 1 PM.

Counts of peripheral blood and bone marrow neutrophils. Blood was collected from the heart of anesthetized rats and each donor was used only once. Total blood neutrophils were determined by diluting the blood 1:200 with Turk's diluting fluid and counting total leukocytes in a hemocytometer followed by a differential count of a smear stained with Wright's stain.

Total bone marrow counts were done on the humerus. It was clipped close to the proximal and distal ends and flushed six times with 2.5 ml of a 0.1% EDTA in sterile saline solution (12). The counts were made in a hemocytometer after diluting 1:20 with Turk's solution.

Differential counts were made from smears of femur marrow stained with Wright's stain. The myeloblasts, promyelocytes, and myelocytes were classified as immature granulocytes. The mature cells were represented by the metamyelocytes, band cells, and polymorphonuclear cells (13). The totals in these cell types were obtained by multiplying the percentage of these cells in the femur times the total cells found in the humerus.

Bone marrow culture. Bone marrow colonies were grown by modification of the method of Broxmeyer *et al.* (14) in McCoy's medium 5a supplemented with 20% fetal bovine serum. Penicillin and streptomycin were added to a final concentration of 0.002%, and this mixture was filtered through a 0.22-μm Millipore filter. Autoclaved agar was cooled to 43° and added to make a final concentration of 0.3%. Finally, 3.33×10^4 freshly prepared rat femoral marrow cells were added/ml, and 3 ml was pipetted into sterile 15 × 60-mm plastic petri plates containing the test material. The plates were incubated for 10 days at 37° in a humidified atmosphere and gassed with 5% CO_2:95% air.

The number of colonies (more than 50

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cells) was counted with the aid of an inverted microscope. Morphological identification was by removal of individual colonies and staining with Wright's stain.

Colony stimulating factor and adherent cells. Excised whole lungs from endotoxin-injected rats were used for the preparation of CSF by the method of Sheridan and Metcalf (15). Increased numbers of adherent cells were obtained by modification of the methods of Messner *et al.* (16). To each petri plate was added 1×10^6 bone marrow cells in McCoy's medium 5a containing 20% fetal bovine serum. These plates were incubated for 30 min at 37°, the supernatant containing nonadherent cells was poured off, and the adherent cells were washed with the incubation media. The bone marrow cells in agar were then poured on top of these adherent cells.

Results. There was a fourfold increase in blood neutrophils 5 hr after the initial injection of 1×10^8 LEM (Fig. 1). Multiple injections resulted in further increases in peripheral blood neutrophils and they remained elevated six- to eightfold after 10 to 30 injections. Total blood leukocytes decreased 5 hr after the first few injections of LEM. This was primarily due to a moderate lymphopenia that seemed to persist throughout the entire series of injections. The increase in total leukocytes occurring after the tenth injection could nearly be accounted for by the increased number of neutrophils. The continued high numbers of blood neutrophils might be expected to exhaust bone marrow reserves, so the maintenance of elevated neutrophils may be an indication that increased granulopoiesis was occurring.

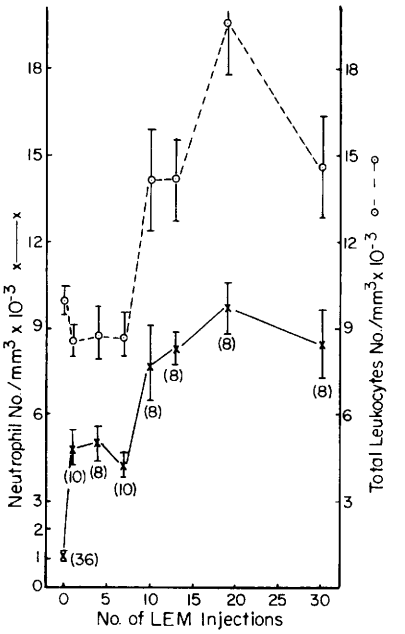

FIG. 1. The effect of multiple injections of LEM on peripheral blood neutrophils. Each rat received the LEM prepared from 1×10^8 granulocytes every 8 hr. The brackets indicate the standard error for the number of rats shown in parenthesis.

The effects of multiple LEM injections on total marrow cells, as well as mature and immature neutrophils, are shown in Table I. The first injection of LEM caused a marked decrease in mature neutrophils which continued to decrease with subsequent injections until the tenth when a recovery occurred. This also was the time when immature neutrophils started to increase. This increased granulopoiesis permitted the mature granulocytes to return to approximately half of their normal numbers by the 30th injection.

At varying times during the repeated injections of LEM, rat serum was collected and checked for CSF (Table II). CSF was detected in the sera from rats receiving four injections and was increased still further by the tenth injection. A check was made, therefore, to see whether varying concentrations of LEM added directly to the agar plates would stimulate colony formation (Fig. 2). When LEM was added in normal rat serum, there was a log dose response with increasing concentrations of LEM. A significant increase in colony formation was observed when LEM from 1000 or more granulocytes was added to a culture plate.

To help explain the effects of LEM on colony formation, CSF or LEM was added to bone marrow cultures in the presence or absence of normal pooled rat serum and with cultures enriched for adherent bone marrow cells (Table III). There was an increased number of bone marrow colonies with added LEM only in the presence of normal rat serum, whereas with CSF rat serum was not required. Very few colonies were observed when adherent cells were in-

TABLE I. BONE MARROW CELLS AFTER REPEATED INJECTIONS OF LEM.

Number of LEM injections	Number of trials	Total cells/humerus $\times 10^{-7}$	Mature neutrophils $\times 10^{-5}$	Immature neutrophils $\times 10^{-5}$
0	36	4.4 ± 0.3[a]	64 ± 6	99 ± 8
1	10	5.0 ± 0.5	16 ± 5*	131 ± 18
4	8	3.8 ± 0.4	8 ± 3*	104 ± 18
7	10	3.1 ± 0.3*	4 ± 2*	115 ± 17
10	8	3.6 ± 0.4	18 ± 4*	187 ± 32**
13	8	3.7 ± 0.3	14 ± 5*	220 ± 38*
19	8	3.3 ± 0.2*	14 ± 4*	177 ± 9*
30	8	2.7 ± 0.3*	30 ± 6*	136 ± 16**

[a] Mean ± SE.
* Significantly different from control: $P < 0.005$.
** Significantly different from control: $P < 0.05$.

TABLE II. COLONY FORMATION OF RAT BONE MARROW CELLS IN AGAR FOLLOWING ADDITION OF 0.2 ml OF SERUM FROM RATS RECEIVING REPEATED INJECTIONS OF LEM.

Number of LEM injections	Number of trials	Colonies/1 × 10^5 cells
0	8	6 ± 2
4	8	19 ± 3*
10	8	43 ± 4*,**
16	8	40 ∓ 2*,**

* Significantly different from zero injections: $P < 0.005$.
** Significantly different from four injections: $P < 0.005$.

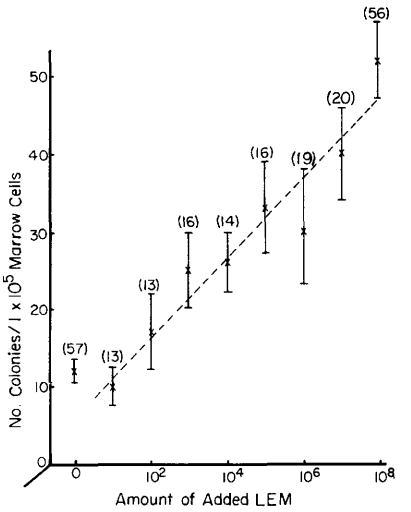

FIG. 2. The effect of LEM upon bone marrow cells in agar culture. Each culture plate, including the controls, contained 0.2 ml of normal rat serum. The brackets show the standard error for the number of trials shown in parenthesis.

cubated without the addition of the other marrow cells. It is quite possible that these few colonies were due to incomplete removal of other cells by only one wash. There was considerable release of CSF by the adherent cells when only rat serum was added. The addition of LEM, however, caused a further stimulation in colony formation, and this stimulation was eliminated when heat-inactivated LEM was used. Adherent cells did not further enhance the effects of CSF. When some of the adherent marrow cells were removed by 30-min incubation at 37°, there were fewer colonies formed in the presence of rat serum and LEM. The removal of these cells had no effect on the stimulation resulting from the addition of CSF. No differences were observed in the morphology of the cells in colonies stimulated by LEM or CSF.

Discussion. Many host alterations have been attributed to LEM following acute infections or inflammation (1–7). A variety of sites of action are involved, and it seems unlikely that LEM could be acting directly on all of them; for fibrinogen synthesis an indirect action has been shown (17). Although further investigations on mechanisms of action will be required, it seems possible that LEM is promoting colony formation by causing the bone marrow mature monocytes to release CSF. This interaction between LEM and the cells which produce CSF in rat bone marrow requires the presence of rat serum, and rat serum alone can promote some release of CSF.

A single injection of endotoxin in mice or rats will cause the release of CSF into the animals blood stream (8–10); but following

TABLE III. STIMULATION OF COLONY FORMATION BY RAT BONE MARROW CELLS WITH LEM OR CSF IN THE PRESENCE OR ABSENCE OF RAT SERUM

Additions to standard culture medium	Number of trials	Colonies/1 × 10^5 cells
None	20	0
LEM[a]	20	1 ± 1[b]
Rat serum[c]	96	22 ± 4
LEM + rat serum	91	70 ± 7
CSF	20	91 ± 5
CSF + rat serum	26	93 ± 11
Adherent cells[d] − bone marrow cells	18	4 ± 1
Adherent cells + rat serum − bone marrow cells	18	14 ± 4
Adherent cells + rat serum + LEM − bone marrow cells	18	12 ± 4
Adherent cells + rat serum	24	78 ± 14
Adherent cells + rat serum + LEM	30	166 ± 17
Adherent cells + rat serum + heated LEM[e]	21	70 ± 10
Adherent cells + CSF	8	85 ± 11
Adherent cells + rat serum + CSF	28	102 ± 12
Nonadherent bone marrow cells[f] + rat serum	8	15 ± 3
Nonadherent bone marrow cells + rat serum + LEM	8	27 ± 5
Nonadherent bone marrow cell + CSF	8	89 ± 18

[a] From 1 × 10^8 granulocytes.
[b] Mean ± SE.
[c] Pooled normal serum (0.2 ml).
[d] The adherent cells from 1 × 10^6 total marrow cells/plate.
[e] 90° for 30 min.
[f] Those cells which did not adhere after 30 min at 37°.

two or more injections of endotoxin, CSF is no longer detectable in the serum (16, 17). Multiple injections of LEM cause greater increases of CSF in serum than a single injection (Table II) and also maintain increased blood neutrophils for extended periods of time (Fig. 1).

Studies by Broxmeyer *et al.* (14) have shown that separate regulators exist for neutrophil production and release. They found that sera obtained 2 hr after endotoxin contained both CSF and the neutrophil release factor; but when multiple injections of endotoxin were given, the CSF was lost but the release factor was retained. It is now known that mice develop a rapid tolerance to endotoxin for CSF (18); but the tolerance to endotoxin for fever, neutrophil release, and LEM develops slowly (18–20). At various times endogenous factors causing fever (21), increased blood neutrophils (22, 23), and numerous host alterations including the two mentioned above (4) have been described. Recent studies with highly purified endogenous pyrogen indicate that it is responsible for all of these activities (11). We prefer the name LEM since it suggests the multiple biological activities of this mediator.

It is now recognized that local production of CSF in bone marrow occurs (24, 25) and that most CSF-producing cells can be removed by adherence to glass or plastic surfaces (14). Although granulocytes were occasionally suggested as a possible source of CSF (8, 26–28), recent evidence indicates that monocytes are the major source (16, 25, 29, 30). The role generally assigned to the granulocyte is inhibition of colony growth (31–33). Granulocytes still may have an indirect role in stimulation of bone marrow leukocytes particularly during infection or inflammation when LEM is being released by these cells. LEM in addition to releasing neutrophils from the marrow (5) may interact with mature monocytes and increase CSF. This mechanism probably has no role during normal granulopoiesis but functions only during stresses which cause the release of LEM (4, 34). It is recognized that there are factors other than LEM which cause monocytes and other cells to release CSF. This was shown in this investigation by the increased colony formation when rat serum was added to adherent cells. To unravel the interrelationship of LEM, serum, and monocytes in CSF production will require a more sophisticated test system than the one we had available for these studies.

Summary. Repeated injections of leuko-

cytic endogenous mediator (LEM) were given every 8 hr to rats for 10 days. Peripheral blood neutrophils were elevated rapidly and after several injections were maintained at six- to eightfold normal levels. Mature bone marrow neutrophils were depressed after the first injection and immature marrow neutrophils increased after the tenth injection. Serum from rats receiving multiple LEM injections had increased amounts of colony-stimulating factor (CSF). Increased bone marrow colony stimulation also resulted when LEM was added directly to agar culture plates of bone marrow cells. This occurred only in the presence of rat serum and the population of adherent bone marrow cells. It was suggested that LEM in the presence of serum acts on mature monocytes to promote the release of CSF.

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