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Mechanisms of Leukocyte Production and Release XII. A Comparative Assay of the Leukocytosis-Inducing Factor (LIF) and the Colony-Stimulating Factor (CSF)[1] (37993)

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Definitive evidence for humoral control of leukocyte release became available when a leukocytosis-inducing factor (LIF) was found in plasmas of rats subjected to repeated leukocytapheresis (1–3). This LIF has been observed to evoke early release of labeled granulocytes from the marrow (4). A neutrophilia-inducing-factor (NIA) has also been found in plasma of dogs treated with vinblastine or nitrogen mustard and has been shown to actually accelerate neutrophil release from the blood-forming organs (5, 6). Until recently, however, it has not been possible to directly test whether these factors are also leukopoietic. With the development of readily reproducible soft agar marrow leukocyte culture techniques (7), it has become feasible to test material for its ability to initiate and support leukocyte production. Some evidence has been presented indicating that materials which stimulate agar colonies *in vitro* (CSF) and granulopoiesis *in vivo* ("Granulopoietin" and "Diffusible Granulopoietic Stimulator") have no releasing activity *in vivo* (8–10). Recently, however, Chervenick (11) has suggested that a single factor may be responsible for regulating neutrophil production and release. The present studies were undertaken to investigate whether or not separate physiological factors do indeed exist for neutrophil production and release.

Materials and Methods. Male rats of a modified Long–Evans strain maintained on Purina Chow and tap water *ad lib.* were used in all experiments.

Perfusion system. A modification of previous perfusion systems was employed (12–16) and preparation of the rat hind leg for perfusion has been described previously (17). The system consisted of a Harvard peristaltic pump (model 600) which propelled oxygenated (arterial) blood from a reservoir, through the cannulated iliac artery into the isolated hind leg of a 250- to 300-g rat and out into a calibrated collecting tube via vena cava return. No reoxygenation of blood was necessary, since only one-way perfusions were conducted. The blood and leg were maintained at 37°C with infrared lamps by a Thermowatch Electronic S-3 controller. The blood flow rate was held at approximately 1.0 ml/min, and perfusion pressures were monitored by a mercury manometer.

Isolated perfused rat hind leg assay. The leg was perfused with leukocyte-depleted blood as previously described (1) until the peak release period was passed (usually attained within the first 30 min of perfusion). At 30 min, 10.0 ml of test sera (and packed red cells to maintain normal hematocrit) was perfused through the leg. An increase in absolute cell count exceeding by at least 50% the highest release during the peak

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control period was taken as a positive test of releasing activity. Since the greatest leukocyte release normally occurred in the first 30 min, an increase of 50% above the highest baseline count of the first 30 min was actually an overall increase of more than 50%; a decrease below this baseline value, however, was not necessarily a decrease below the release that would have occurred after 30 min of perfusion. Using the first 30 min as a baseline for activity made it possible to test release activity in each individual perfusion. Two perfusions were performed for each assay group and each perfusion tested sera prepared on different days.

Agar culture system. Femoral and tibial bone marrow cells from 150-g male rats were cultured in soft agar medium at concentrations of 1×10^5 cells/ml using a modification of previous techniques (18). The medium consisted of equal parts of a 0.6% agar preparation with double-strength Eagle's modified medium containing 5% heat-inactivated newborn calf serum, 5% bovine serum, 10% soy broth, 1-asparagine (final concentration 20 μg/ml), and DEAE–dextran (final concentration 75 μg/ml). The test serum (0.1 or 0.2 ml) was placed in 60 × 15-mm Falcon plastic gridded culture plates to which 3.0 ml of bone marrow cell suspension in agar medium was added. The plates were thoroughly mixed, and allowed to gel at room temperature before being incubated at 37°C in humidified atmosphere and gassed with 5% CO_2 in air for 10 days. For each assay sample three to five plates were prepared.

In vivo releasing factor assay. The assay formerly performed in this laboratory (2, 19) was utilized in slightly modified form. A single intraperitoneal (ip) injection of 1.0 ml of concentrated test serum (3.0 ml of serum lyophilized and reconstituted to 1.0 ml) was given to recipient 150- to 200-g rats divided into three groups of five to eight rats each (see below).

Preparation of sera. Serum was obtained from the dorsal aorta of ether-anesthetized 200-g male rats. The following sera were tested: Group I, control serum from untreated rats with leukocyte tail counts of 10,000–18,000 cells/mm^3; Group II, serum collected 2 hr after intravenous (iv) administration of 4.0 μg *E. coli* lipopolysaccharide 026:B6 (Difco Laboratory, Detroit, MI) dissolved in 0.5 ml of sterile pyrogen-free saline. Group III, serum collected 27 hr after the last of five daily ip injections of 100.0 μg of *E. coli* lipopolysaccharide in 20.0 ml of sterile pyrogen-free saline. Group IV, serum collected 27 hr after the last of 2 daily ip injections of 100.0 μg *E. coli* lipopolysaccharide in 20.0 ml of sterile pyrogen-free saline. Group V, *E. coli* lipopolysaccharide added to normal serum in concentrations of 0.1–100.0 μg per total test volume prior to hind leg perfusion. All sera were filter sterilized through a 0.45-μ Millipore filter. Groups I–V were tested in the isolated perfused rat hind leg assay. Groups I–IV were tested in the agar culture system. Groups I–III were tested in the *in vivo* releasing-factor assay.

Test for endotoxin contamination in test sera. Spontaneous increases in perfusion pressures during isolated hind leg perfusions are usually indicative of high endotoxin contamination (16). The Etoxate test (Sigma Chemical Co.), sensitive to 1×10^{-4} μg of *E. coli* endotoxin, was used to test for such contamination (20). As anticipated, 2.5 μg of *E. coli* lipopolysaccharide gave a distinct positive reaction for Etoxate (gelation within 15 min), and 0.25 μg produced gelation by 30 min. However, no test sera gave a positive Etoxate reaction, even after 30 hr. Moreover, while 100 μg of endotoxin caused spontaneous increases in perfusion pressures, none of the test sera had this effect.

Results. Table I demonstrates that Group II sera manifested colony-stimulating activity (CSA) at doses of 0.1 and 0.2 ml. In addition, a dose-response effect was suggested as 0.1 and 0.2 ml, respectively, stimulated 128.0 ± 24.9 and 363.0 ± 9.8 colonies/1×10^5 bone marrow cells.

Table II summarizes the effects of the various sera when administered to intact rats. Group I sera showed no releasing activity with actually a significant decrease in granulocytes noted by 24 hr. Group II sera, which contained CSA, evoked a significant granulocytosis at 1, 4, and 7 hr posttreat-

TABLE I. Effects of Test Rat Sera on the Numbers of Agar Colonies Formed.[a] Group I: Sera from Untreated Rats; Group II: Sera Obtained 2 hr Post-iv Injection of 4.0 μg *E. coli* Lipopolysaccharide into Rats; Group III: Sera Obtained 27 hr After the Last of Five Daily ip Injections of 100.0 μg *E. coli* Lipopolysaccharide; Group IV: Sera Obtained 27 hr After the Last of Two Daily ip Injections of 100.0 μg *E. coli* Lipopolysaccharide.

	Dosage (ml)	Agar colonies[b] 1×10^5 bone marrow cells
Group I	0.2	7.25 ± 2.25[c]
Group II	0.1	128.0 ± 25.9
	0.2	363.0 ± 9.8
Group III	0.1	10.0 ± 5.8
	0.2	6.7 ± 1.7
Group IV	0.1	0
	0.2	3.3 ± 1.7

[a] Each petri dish initially contained 3.0×10^5 rat bone marrow cells/3.0 ml medium.
[b] Cell aggregates containing at least 50 cells/colony.
[c] Mean ± SEM values of four replicate cultures.

ment with total white blood cell levels diminished at 7 hr and mononuclear numbers decreased at 4 and 7 hr. The serum of Group III also showed releasing activity, producing a significant granulocytosis at 4 and 7 hr. Group IV serum was not tested in the *in vivo* assay.

Isolated perfused hind leg assay data are

TABLE II. Effect of Test Rat Sera on PMN, Mononuclear, and Total White Cell Counts of Rats. Group I: Effects of a Single 1.0-ml Injection of Test Sera Obtained from Untreated Rats; Group II: Effects of a Single 1.0-ml Injection of Test Sera Obtained 2 hr Post-iv Injection of 4.0 μg *E. coli* Lipopolysaccharide; Group III: Effects of a Single 1.0-ml Injection of Sera Obtained 27 hr After the Last of Five Daily ip Injections of 100.0 μg *E. coli* Lipopolysaccharide.

Hr post-injection	White cell count (cells/mm³)	Polymorphonuclear (cells/mm³)	Mononuclear[a] (cells/mm³)
0 (18)[b]	14989 ± 663[c]	2522 ± 146	12236 ± 603
Group I			
1 (8)	15080 ± 1271	3524 ± 912	11277 ± 1191
4	18560 ± 2896	4123 ± 955	14237 ± 2315
7	15580 ± 1135	3826 ± 763	11597 ± 849
24	13570 ± 1096	1691 ± 351[d]	11634 ± 888
Group II			
1 (5)	16130 ± 1719	5287 ± 979[e]	10740 ± 972
4	15010 ± 942	5228 ± 748[f]	9782 ± 541[e]
7	12360 ± 725[e]	3743 ± 515[d]	8597 ± 665[g]
24	16380 ± 877	2077 ± 380	14134 ± 903
Group III			
1 (5)	15330 ± 2310	2860 ± 911	12471 ± 2191
4	16440 ± 2395	4461 ± 496[f]	11954 ± 2098
7	14240 ± 1325	4104 ± 564[e]	10121 ± 1124
24	15830 ± 1735	3372 ± 1226	12232 ± 1372

[a] Mononuclear cells counted were usually more than 95% lymphocytes.
[b] Figures in parentheses indicate the numbers of animals in each group.
[c] Each count represents the mean ± SEM.
[d] $P \leq 0.05$ when compared to the preinjection level.
[e] $P \leq 0.02$ when compared to the preinjection level.
[f] $P \leq 0.005$ when compared to the preinjection level.
[g] $P \leq 0.001$ when compared to the preinjection level.

TABLE III. Effects of 10.0 ml of Test Rat Sera or Various Concentrations of *E. coli* Lipopolysaccharide in Perfused Rat Hind Leg Assay. Group I: Sera from Untreated Rats; Group II: Sera Obtained 2 hr Post-iv Injection of 4.0 μg *E. coli* Lipopolysaccharide; Group III: Sera Obtained 27 hr After the Last of Five Daily ip Injections Each of 100.0 μg *E. coli* Lipopolysaccharide; Group IV: Sera Obtained 27 hr After the Last of Two Daily ip Injections of 100.0 μg *E. coli* Lipopolysaccharide; Group V: 0.1–100.0 μg *E. coli* Lipopolysaccharide in Normal Sera.

	Highest % change PMN cells
Group I	5
	−39
	28
Group II	82
	77
Group III	73
	214
Group IV	80
	222
Group V	
0.1 μg *E. coli* lipopolysaccharide	6
1.0 μg *E. coli* lipopolysaccharide	−6
	−66
	−14
	25
50 μg *E. coli* lipopolysaccharide	8
100 μg *E. coli* lipopolysaccharide	−4

presented in Table III. No sera tested demonstrated mononuclear releasing activity. Control sera and 0.1–100.0 μg *E. coli* lipopolysaccharide suspended in control sera prior to perfusion (Group V) showed no granulocyte-releasing activity, but Groups II, III, and IV sera demonstrated a greater than 50% increase in granulocyte release within 20 min after the test material was added to the perfusion system.

Discussion. It is evident that the releasing effects of all active sera tested were not due to endotoxin contamination, since there were no perfusion pressure effects and Etoxate tests were negative. Also, 0.1–100.0 μg *E. coli* lipopolysaccharide suspended in control sera prior to perfusion showed no leukocyte-release activity during the test period. Removal of endotoxin by body tissues is rapid. Over 90% of ^{51}Cr-*E. coli* endotoxin was found to be cleared from the peripheral blood of rabbits within 5 min (21). There has also been no noticeable plasma LIF activity until 3 hr after treatment of rats with typhoid–paratyphoid vaccine (22). Foreign or toxic substances in the sera can also be ruled out, since control sera demonstrated no significant release activity. Thus, it would seem unlikely that the total white blood cell decrease at 7 hr and the fall in mononuclear cells seen at 4 and 7 hr with Group II sera was due to endotoxin contamination. The drop in total white blood cell numbers at 7 hr was due to the diminution seen in mononuclear cell levels.

Since sera shown to be active *in vivo* were also active on the isolated rat hind leg assay, the value of 50% above the highest count of the first 30 min was a valid indication of release. It is impossible at the present time to quantify the releasing effects of active sera using the isolated rat hind leg assay. Thus, although it appears that some sera are more active than others, more experiments will be needed to rule out variability due to the individual responses of isolated hind legs.

The active releasing factor present in Groups II, III, and IV sera should probably be termed a neutrophilia-inducing factor (3), as it seems to be specific for

neutrophilic granulocyte release. These experiments also indicate that the various groups of sera have differing effects on neutrophil production and release. For example, Group II serum stimulated both production and release, whereas Group III and IV sera (Group IV sera was tested only in perfusions and in agar culture), showed only releasing activity. As for the dual action of Group II serum, it is possible that one treatment induced the production and/or activation of both an LIF and a CSF. If both activities were attributed to a common humoral principle, then any treatment eliciting LIF should also show CSA. That this was not the case, is shown from Group III and IV sera data, which stimulated release only.

Control sera (Group I, Table II), produced increases in PMNs which were not significant statistically. This may indicate the presence of low quantities of NIF in the control group sera. If NIF is a physiological regulator, it would be expected that untreated animals could occasionally exhibit low but significant circulating levels of the factor.

The dosage and time intervals found to elicit CSF and LIF were adequate to show that they were separate entities. However, tests of other schedules are in progress, since it is obvious from the data that several dosage and time intervals will elicit LIF activity.

Other workers have already shown that CSF does not directly promote leukocyte release. For example, purified human urine CSF has no releasing activity (8) nor does the granulopoietic stimulating factor induced in mouse plasma 18 hr post-endotoxin administration (9). Recently a time sequence study in mice given 40.0 μg *Salmonella* endotoxin demonstrated the presence of a plasma-borne neutrophil-releasing factor within 4 hr, while a diffusible granulopoietic stimulator was not noted until 48 hr later (10). On the other hand, Chervenick (11) has suggested that since a neutrophil-releasing factor and a CSF appeared concomitantly after endotoxin and vinblastine (VLB) treatment, the two factors are really the same principle. However, such a finding could also be interpreted to mean that endotoxin and VLB treatment induced two different factors in the same media, as appears to be the case for Group II sera of this experiment.

Clearly, it will be necessary to chemically purify and characterize these factors before it can be established with certainty whether or not they are separate entities. This may, however, prove to be difficult especially if the factors are chemically and/or physically similar. Studies on the purification of LIF carried out by Katz *et al.* (3) have shown LIF to be a thermolabile, nondialyzable pseudo-α-globulin. Katz compared his LIF to other factors found to cause release. Some were chemically similar, whereas others were not. CSF is present in serum, urine, and many organ extracts in different physical and chemical forms (23). Human urine CSF has a molecular weight of about 45,000 and is a neuraminic acid containing glycoprotein (24, 25). Given the many possible forms of CSF, it may be impossible to distinguish them from the LIF, which may vary in a similar way. Further work is presently being carried out in this laboratory to more clearly define the physiological and chemical properties of the LIF.

Summary. Experiments are reported which suggest that separate physiological regulators exist for neutrophil production and release. Sera obtained 2 hr post-iv injection of 4.0 μg *E. coli* lipopolysaccharide contained both LIF and CSF activity, whereas sera obtained 27 hr after the last of two and the last of five daily ip injections of 100.0 μg *E. coli* lipopolysaccharide demonstrated only LIF activity. Using the Etoxate test, it was established that neither LIF nor CSF activity could be attributed to endotoxin contamination of test sera.

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