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Comparison of Neutrophilia Induced by Leukocytic Endogenous Mediator and by Cobra Venom Factor (41349)

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Abstract. Neutrophilia is an important aspect of acute inflammation. We have compared the neutrophilia induced by cobra venom factor (CVF) activation of complement with the neutrophilia produced by injection of leukocytic endogenous mediator (LEM) to determine if interrelationships exist. Study of peripheral blood neutrophils and bone marrow neutrophil release indicate different kinetics for neutrophilia following LEM as compared to that following CVF. In addition, LEM did not activate plasma complement to produce a neutrophilia factor and CVF-activated complement split-products, capable of inducing neutrophilia, did not show the LEM activities of changes in plasma iron or plasma fibrinogen.

The increase in number of peripheral blood neutrophils is an important aspect of acute inflammation. Leukocytic endogenous mediator (LEM), a 13,000- to 15,000-dalton protein (1–3) synthesized by macrophages (4, 5), promotes release of neutrophils from bone marrow (6–9). A delay of 30 or more minutes occurs before the number of peripheral blood neutrophils increases after various doses of LEM (9). This delay may indicate that LEM acts through some other mediator. Since complement has been shown to effect the number of peripheral blood leukocytes (10–18), a logical possibility for interrelationship exists.

Complement activation *in vivo* by cobra venom factor (CVF) causes leukocytosis in several species (10, 11, 13, 14, 18). A possible mechanism considering LEM as a complement activator can be investigated by comparing kinetics of peripheral blood neutrophilia and bone marrow neutrophil releasing activity after LEM or CVF and by incubating LEM or CVF *in vitro* with plasma.

The particular product of complement activation responsible for leukocytosis has not been established with the exception of C3e (15). C3e, with a molecular weight similar to LEM, has been shown to increase rabbit peripheral blood leukocytes (15) suggesting the alternate possibility that LEM is a complement split-product, particularly since macrophages are known to synthesize complement components (19–22). LEM, in addition to causing neutrophilia, causes a variety of other biological activities such as fever (1, 2, 23), changes in plasma metals (24–29), and changes in acute phase proteins (30–34). The split-products from *in vitro* activation of complement by CVF, known to induce neutrophilia, were investigated for two well-studied LEM activities, i.e., change in the concentrations of plasma iron and plasma fibrinogen.

Data presented suggest two apparently different factors, one elaborated by the reticuloendothelial system (LEM) and one by the complement cascade, each producing a neutrophilia with different kinetics.

Materials and Methods. *Animals.* Holtzman-derived 8- to 10-week-old rats weighing approximately 200–230 g each were bred in our colony.

Reagents. Cobra venom factor (*Naje haje*) was obtained from Cordis Laboratories, Miami, Florida, and typically had 100 anticomplementary units/ml, a protein content of 0.07 mg/ml, and 0 lecithinase (Cordis assays). CVF was assayed by the spectrophotometric *Limulus* assay (35) and no endotoxin contamination was found. In studies to be published we have shown that formation of the neutrophilia factor in plasma was blocked or reduced by heat inactivation (56°) of the plasma or addition of EDTA. Sodium heparin, 1000 U/ml (Lilly, Indianapolis, Ind.), was used at 20 U/ml final concentration for anticoagulation. Pyrogen-free saline was obtained from Travenol, Deerfield Park, Illinois. Metofane in-

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halation anesthetic (Pitman-Moore, Washington Crossing, N.J.) was used.

Direct injection of CVF. Twenty units CVF was injected into the femoral vein of anesthetized rats. Reanesthetized animals were bled by cardiac puncture with 20 U heparin in plastic syringes.

In vitro incubation of CVF with plasma. Plasma was obtained from heparinized blood from cardiac punctures. CVF was incubated at a dose of 5 U/ml rat plasma for specified lengths of time in a 37° shaking water bath. All samples of CVF/plasma for assay were 1 ml injected iv in the femoral vein.

LEM preparation and partial purification. The special procedures for LEM production are detailed elsewhere (8, 24, 25). Source of LEM was rabbit 18-hr glycogen/saline peritoneal exudate leukocytes. Partial purification of crude LEM was achieved on Sephadex G-50 (Pharmacia, Piscataway, N.J.). Detailed procedures are found in previous descriptions (36). LEM was assayed for pyrogenic activity by fever determination in trained New Zealand white rabbits.

Neutrophil assay. Heparinized blood from cardiac puncture was diluted 1/100 in Unopettes (Becton Dickinson 5855, East Rutherford, N.J.) and total leukocytes counted by standard hemacytometer techniques. Differential counts were made on Wright's-stained smears. Total neutrophils/mm^3 were calculated by multiplying total WBC/mm^3 by the percentage of neutrophils.

Bone marrow neutrophil assay. The total number of cells in the humerus was determined as previously described (37). Bone marrow smears from the femur were Wright's stained and mature neutrophil percentage determined by differential counts on 500 cells. The total number of mature neutrophils in the humerus was calculated by assuming that the differential count in the humerus would be the same as that observed in the femur (9).

LEM injected into C3-depleted rats. Rats were depleted of antigenic C3 by iv injection of 40 U CVF. Rocket immunoelectrophoresis (38) was done on plasma samples taken at the time of neutrophil assay. Monospecific rabbit anti-rat C3 (Cappell Labs, Cochranville, Pa.) was used. Pooled, normal rat serum was analyzed as standards for C3 and it was determined that dilutions of 1/50 still gave measurable rockets when stained with Crowle's double stain (39). All rats said to be C3-depleted showed no rocket-measurable C3, i.e., they had less than 2% of the antigenic C3 in pooled, normal rat serum. Saline-injected controls, however, uniformly had C3 rockets no different in peak height than the pooled normal standard. LEM, 0.5 pyrogenic unit, was injected iv 24 hr after CVF injection for C3 depletion. One hour after LEM injection neutrophil assays were done on heart blood.

LEM incubation with plasma. LEM, 0.5 pyrogenic unit/ml plasma, was incubated for 30 min in a 37° shaking water bath. Neutrophil assays were done 10 min after iv injection of 1-ml samples.

Plasma iron assay. Heparinized plasma was obtained from rats 8 hr after injection of samples. Plasma iron levels were determined according to the technique of Shade (40).

Fibrinogen assay. Heparinized plasma was obtained from rats 24 hr after injection of samples. Plasma fibrinogen levels were determined according to the technique of Wycoff (41).

Statistics. All values represent the mean ± the standard error of the mean with the number of values in parentheses. Statistical significance, where appropriate, was determined by the Student's t test. Linearity was determined by least-squares fit.

Results. Kinetics of the leukocytosis after injection of CVF, CVF/plasma, or LEM were compared to investigate whether LEM might activate complement. We established the magnitude and the time course for the leukocytosis induced by direct iv injection of CVF and found it to be a neutrophilia (Fig. 1). A mild and brief neutropenia was noted at 5 and 10 min after injection. Neutrophilia began by 20 min and continued with a linear increase ($r = 0.998$) through 30 min to 60 min. The number of neutrophils/mm^3 at 60 min represented a 12.7-fold increase over controls.

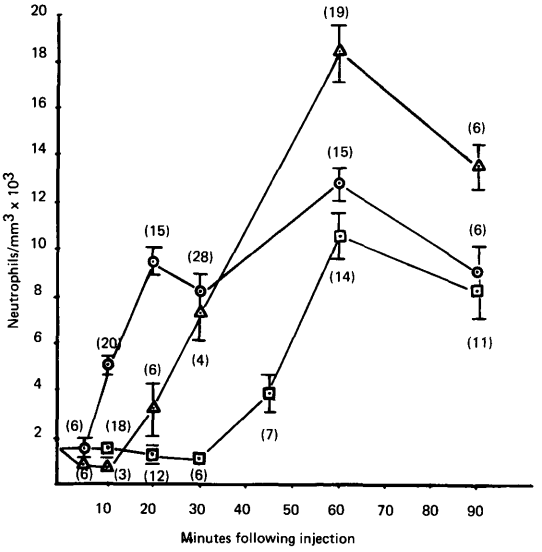

FIG. 1. Neutrophilia in rats following iv injection of cobra venom factor (CVF) △, 1 ml of rat plasma incubated with 5 U CVF *in vitro* for 45 min at 37° (CVF/plasma) ○, or 1 ml leukocytic endogenous mediator (LEM) □. Shown are means ± SEM of total neutrophils/mm³ in heart blood with number of samples in parentheses.

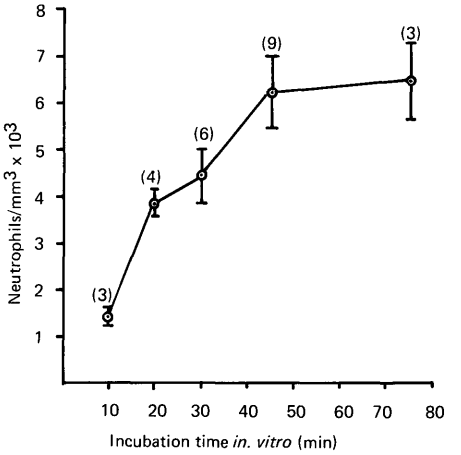

FIG. 2. Various *in vitro* incubation times of cobra venom factor with rat plasma (5 U/ml) and the resulting neutrophilia 10 min after iv injection of a 1-ml sample. Shown are means ± SEM of total neutrophils/mm³ in heart blood with number of samples in parentheses.

Injection of CVF/plasma produced an earlier increase in neutrophils than was seen after CVF. A definite neutrophilia began by 10 min and continued sharply to 20 min followed by a more gradual rise up to 60 min.

Figure 1 also shows the neutrophilia kinetics for LEM. No increase in peripheral neutrophils was seen at 10, 20, or 30 min after injection. By 45 min after 1.0 pyrogenic unit LEM was injected a neutrophilia began and by 60 min LEM caused an approximately eightfold increase over controls.

Figure 2 demonstrates the time required for maximum neutrophilia factor generation. The 5 U CVF/ml rat plasma approximated the direct iv dose of 10 U CVF/100 g body weight. The assay time of 10 min after injection was chosen so any carryover of CVF would not interfere (Fig. 1). Twenty minutes of *in vitro* incubation were needed to generate significant neutrophilia upon reinjection of 1 ml CVF/plasma into normal rats. This correlates well with 20 min being the first sign of neutrophilia following CVF injected directly iv. Maximum generation of neutrophilia factor *in vitro* occurs by 45 min of incubation as further incubation to 75 min did not result in additional factor generation (Fig. 2).

Further investigation of possible LEM and complement interrelationship is contained in Table I. Injection of 1 ml CVF/plasma produced both neutrophilia and depletion of mature neutrophils from the bone marrow at 10 min (Table I) indicating the bone marrow as a source of neutrophils found in the peripheral blood. The magnitude of neutrophilia at 60 min caused by direct iv injection of CVF was much greater than that caused by CVF/plasma, and the bone marrow data reflect this difference. At 60 min CVF injected directly iv and LEM alone both produced neutrophilia and bone marrow depletion (Table I), yet despite similar bone marrow values the number of peripheral neutrophils/mm³ was nearly twofold higher in rats injected with CVF. These data suggest that the bone marrow may not be the only source of the increased number of peripheral neutrophils seen after CVF or CVF/plasma injection.

In addition to comparison of neutrophilia kinetics we determined that LEM was able to induce the same magnitude neutrophilia

TABLE I. COMPARISON OF NEUTROPHIL LEVELS IN PERIPHERAL BLOOD AND IN BONE MARROW AFTER IV INJECTION OF COBRA VENOM FACTOR (CVF) OR LEUKOCYTIC ENDOGENOUS MEDIATOR (LEM)

	Peripheral blood[a]		Bone marrow[b]	
	10 min	60 min	10 min	60 min
Plasma control[c]	1812 ± 318 (12)	1,490 ± 155 (12)	7.04 ± 0.77 (12)	7.32 ± 0.96 (12)
CVF/plasma	4479 ± 426 (12)***	12,386 ± 636 (12)***	3.78 ± 0.59 (12)**	4.82 ± 0.94 (12)
LEM in plasma	1853 ± 351 (12)	10,767 ± 972 (11)***	6.06 ± 0.72 (12)	2.56 ± 0.40 (11)***
Saline control[d]	1469 ± 221 (12)	1,694 ± 244 (18)	7.77 ± 0.91 (12)	8.89 ± 0.89 (18)
CVF	753 ± 187 (3)	19,186 ± 999 (12)***	7.81 ± 1.21 (3)	2.92 ± 0.48 (12)***
LEM	1597 ± 256 (18)	10,305 ± 3177 (3)***	n.d.[e]	3.06 ± 0.48 (3)*

[a] Mean total neutrophils/mm^3 ± SEM (number of values).
[b] Mean total mature neutrophils/humerus × 10^{-6} ± SEM (number of values).
[c] 1 ml plasma as comparison for 1-ml sample of 5 U CVF incubated 45 min, 37°, with 1 ml plasma (CVF/plasma) and for 1.0 pyrogenic unit LEM contained in 1 ml plasma (LEM in plasma).
[d] 1 ml nonpyrogenic saline as comparison for 20 U CVF in 1 ml saline and for 1.0 pyrogenic unit in 1 ml saline.
[e] Not done.
* $P < 0.02$.
** $P < 0.005$.
*** $P < 0.001$.

in rats depleted of antigenic C3, 8896 ± 917 ($n = 10$), as in rats not depleted of antigenic C3, 9320 ± 955 ($n = 8$).

Further evidence that the neutrophilia produced by LEM was not due to complement was found when LEM was incubated for 30 min *in vitro* with plasma. No neutrophilia factor was detected in a 10-min neutrophilia assay, 925 ± 148 ($n = 10$), whereas CVF incubated for 30 min *in vitro* with plasma gave an approximately threefold increase in neutrophils, 4359 ± 597 ($n = 6$), 10 min after injection when compared to plasma controls, 1319 ± 316 ($n = 7$).

An alternate possibility tested was that CVF activation of the alternate pathway of complement may have produced a split-product similar to or identical to LEM. Two well-established biological activities of LEM were examined (Table II). Whereas LEM and LEM injected in plasma markedly lowered plasma iron as compared to plasma controls, CVF/plasma did not. Similarly, LEM elevated plasma fibrinogen; however, CVF/plasma did not when compared to plasma controls.

Discussion. A delay of at least 30 or more minutes before neutrophilia occurs following LEM injection has been previously reported (7, 9) and has been demonstrated in data presented here. This delay may suggest that LEM acts through some other mediator. The complement system has been implicated in leukocytosis responses; therefore, we investigated the possible interrelationships between LEM and complement with respect to induction of neutrophilia, considering LEM as either an activator of complement or as a product of complement activation.

No evidence was found for LEM as an activator of complement. CVF was able to activate plasma complement to generate a neutrophilia factor *in vitro;* however, LEM was not. Analysis of peripheral blood neutrophil increases and corresponding bone marrow losses show good correlation for

TABLE II. BIOLOGICAL ACTIVITIES OF LEM AND CVF/PLASMA COMPARED

	Plasma iron (μg%)	Plasma fibrinogen (mg%)
Plasma control[a]	330 ± 19 (12)[b]	238 ± 9 (9)
LEM	101 ± 5 (5)**	334 ± 11 (9)**
LEM in plasma	188 ± 19 (8)**	272 ± 12 (8)*
CVF/plasma	338 ± 15 (8)	232 ± 9 (17)
Normal rats	322 ± 14 (8)	185 ± 5 (8)**

[a] 1 ml plasma as comparison for 1.0 pyrogenic unit leukocytic endogenous mediator (LEM); for 1.0 pyrogenic unit in 1 ml plasma; and for 1 ml sample of 5 U cobra venom factor (CVF) incubated 45 min, 37°, with 1 ml plasma (CVF/plasma).
[b] All values = mean ± SEM (number of values).
* $P < 0.05$.
** $P < 0.001$.

LEM (9). We have demonstrated that bone marrow depletion of mature neutrophils occurs after CVF or CVF/plasma injection; however, CVF causes greater peripheral neutrophil increases than LEM does when bone marrow neutrophil losses are similar. Furthermore, when CVF and LEM cause similar increases in peripheral neutrophil levels the CVF-treated animals have significantly smaller losses of mature bone marrow neutrophils. These comparisons suggest that neutrophilia induced by CVF has an additional source of neutrophils other than the bone marrow, most likely the marginal pool. If LEM-induced neutrophilia relied on complement activation, we would not expect to find these differences in correlating peripheral neutrophil increases with bone marrow losses. Furthermore, LEM was able to induce neutrophilia in rats depleted of antigenic C3.

Complement activation products do not appear to be more proximal mediators of neutrophilia induced by LEM. Despite the ability to induce neutrophilia, the *in vitro* CVF-activated complement split-products do not have either plasma fibrinogen-increasing or plasma iron-lowering activities, two well-studied biological activities of LEM. This suggests that LEM is not directly a product of complement activation. If LEM were a split-product of complement activation, we would expect it to induce neutrophilia earlier than CVF activation of complement; however, our data indicate that LEM-induced neutrophilia occurs considerably later than that induced by CVF.

Previous investigations of CVF injection in rabbits showed a neutropenia immediately with a return toward normal in 1 (14) to 2 (13) hr. The neutropenia we observed following CVF was mild and transient with significant neutrophilia occurring by 20 min after injection. No neutropenia occurred after LEM injection.

We demonstrated a mean neutrophilia at 60 min following CVF iv that was 13 times the control mean. This increase was much greater than any other neutrophilia values reported for CVF at any time interval or dose in mice (18), rabbits (13), or guinea pigs (11). Gilbertsen *et al.* (17) concluded that rats may be particularly useful for studying neutrophilia, a conclusion corroborated by our results.

LEM does not activate complement to produce neutrophilia nor is LEM directly a split-product of complement activation by CVF. LEM and CVF induce neutrophilias with different kinetics, CVF-induced neutrophilia occurring earlier, with greater magnitude, and with a neutrophil source in addition to the bone marrow. These data suggest that two different neutrophilia factors can be elaborated as host defense responses in infectious disease and inflammation, one by the reticuloendothelial system (LEM) and one by the complement system.

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