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Effect of Human Monocyte Pyrogen on Plasma Iron, Plasma Zinc, and Blood Neutrophils in Rabbits and Rats (40133)

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The pyrogen from rabbit peritoneal granulocytes has been shown to produce a variety of other effects when injected into rabbits or rats (1–3). This factor has been named leukocytic endogenous mediator (LEM) (4); and in addition to granulocytes it can be produced by macrophages, neutrophils, and Kupffer cells (5).

Recently it was shown that following incubation with heat-killed staphylococci, human monocytes produced 10–20 times as much pyrogenic activity as an equal number of neutrophils (6, 7). It was further shown that the monocyte pyrogen has distinctive physical and chemical characteristics; among these was a higher molecular weight of approximately 38,000 Daltons (7). The pyrogens from human leukocytes have been reported to produce fever in rabbits (6–8). Our purpose was to determine whether human pyrogen can produce LEM-like changes in rats and rabbits and, if these changes are observed, will they be greater with the monocyte-derived LEM than has previously been observed with LEM obtained from granulocytes.

Materials and methods. All equipment, media, and reagents were sterile and pyrogen free. Supernatants were checked for bacterial contamination by culture in nutrient broth and thioglycollate and for pyrogenicity by injections into rabbits.

Blood monocytes. Human blood obtained from three healthy male volunteers was collected in a flask with an equal volume of saline containing 100 units/ml of heparin. Mononuclear cells were isolated from the blood-saline mixture on Ficoll–hypaque gradients by the method of Boyum (9). The average recovery of monocytes for each 100 ml of blood was 5×10^7 contaminated with less than 1×10^6 granulocytes.

Pyrogen production. The isolated mononuclear cells were suspended in Hanks' balanced salt solution (HBSS) with 100 units of penicillin, 8 μg of gentamycin, 2 units of heparin per ml, and 10% autologous serum. Heat-killed *Staphylococcus aureus* was added to give a final bacteria to leukocyte ratio of 40 to 1. This suspension was incubated for 60 min in a shaking water bath at 37°. Controls were treated the same way, but without the addition of *S. aureus.* The cells were recovered by centrifugation at 750 g for 10 min, resuspended in serum-free HBSS at a concentration of 1×10^7 cells/ml, and incubation was resumed in a stationary incubator for 18 to 20 hr at 37°. The cells were pelleted at 5000 g for 10 min and the supernatant (crude monocyte pyrogen) was immediately assayed or concentrated three- to fourfold by evaporation at room temperature (7) and fractionated on a gel column.

Column chromatography. The concentrated sample of crude monocyte pyrogen was fractionated by slight modifications of the method of Dinarello *et al.* (7). A 20 ml sample was applied to a 171 × 3.2 cm column of Sephadex G-50 (fine). The column was eluted with 0.15 *M* NaCl at room temperature with a flow rate of 60 ml/hr, and 10 ml fractions were collected. The marker proteins, ovalbumin, chymotrypsinogen A, and Cytochrome C, were used prior to each run and some of the eluted fractions were tested for contaminating pyrogens.

In preliminary runs individual fractions were assayed and pyrogenic activity was observed only at elution volumes corresponding to molecular weights between 36,000 and 40,000 Daltons and also at a molecular weight between 12,000 and 18,000. To compare these two pyrogens we formed two arbitrary pools, one with a midpoint at the 38,000 mol wt elution volume and the other with a midpoint at the 15,000 mol wt elution volume as determined from the marker proteins. The 38,000 pool contained 50 ml (25 ml either side of the midpoint) and the 15,000 mol wt pool contained 100 ml (50 ml either side of the mid-

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point). Separate pyrogen assays were made upon each pool and on either side of and between these pools.

Animals. Female Holtzman-derived rats weighing 160–220 g were fed Rockland rat diet and water *ad libitum.* New Zealand white rabbits weighing 2–3 kg were purchased locally.

Biological assays. Pyrogenic activity was determined in restrainer-trained rabbits whose base line temperatures had stabilized. The dose of pyrogen sufficient to cause 1° fever was determined by measuring rectal temperatures every 5 min and constructing a log dose plot of the fever peak which occurred 40–65 min after injection. Each supernatant was assayed with a minimum of three doses using at least three rabbits per dose.

Plasma iron and zinc concentrations and blood neutrophils were determined 8 hr after injection. Iron and zinc were measured in an atomic absorption spectrophotometer after precipitating the plasma proteins with an equal volume of 20% trichloroacetic acid.

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.

Results. Crude supernatants derived from stimulated human blood monocytes demonstrated LEM activity by inducing granulocytosis and lowering plasma iron and zinc in both rabbits and rats (Table I). Control supernatants from monocytes not stimulated with heat-killed *S. aureus* were inactive (not shown in Table I). The lowering of plasma iron and zinc and the increase in blood neutrophils were determined in animals that received a dose sufficient to cause 1° fever in a rabbit. This dose resulted in more than 100 μg/100 ml decrease in plasma iron and an increase of about 4000 neutrophils/mm^3 in both rats and rabbits. The decrease observed in plasma zinc was larger in rabbits than in rats.

The concentrated samples of crude monocyte pyrogen were fractionated on Sephadex G-50 as shown in Fig. 1. Pyrogenic activity was eluted in the fraction which indicated an approximate molecular weight of 38,000 Daltons. In addition, there was a still larger pool of pyrogenic activity eluted at a molecular weight of 13,000–15,000 Daltons. Tests for activity from fractions eluted at the void volume and between the two peaks gave very little pyrogenic activity.

Four different preparations of supernatants

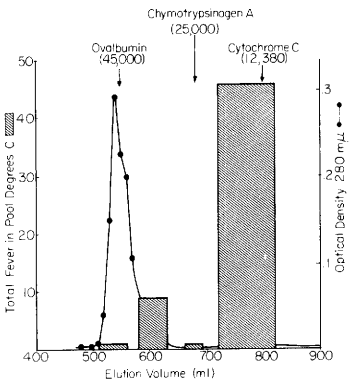

FIG. 1. A typical run of concentrated crude monocyte pyrogen through Sephadex G-50. The column was 171 × 3.2 cm; solvent, 0.15 *M* NaCl; flow rate, 60 ml/hr; sample size, 20 ml. The marker proteins used are shown at the top of the figure. The total fever was calculated by determining the volume of each pool which would produce 1° fever and dividing this into the total volume of the pool.

TABLE I. EFFECT OF INJECTING CRUDE HUMAN MONOCYTE PYROGEN ON PLASMA IRON, PLASMA ZINC, AND BLOOD NEUTROPHILS IN RABBITS AND RATS.[a]

	Rabbits		Rats	
	Control[b]	Injected	Control[b]	Injected
Number of animals	10	6	20	15
Plasma iron μg/100 ml	193 ± 9[c]	86 ± 4[d]	227± 8	110 ± 7[d]
Plasma zinc μg/100 ml	234 ± 8	158 ± 15[d]	116 ± 3	83 ± 4[d]
Neutrophils no./mm^3 × 10^{-2}	16 ± 1	59 ± 7[d]	12 ± 1	52 ± 3[d]

[a] Dose injected was sufficient to cause a 1° fever in rabbits.
[b] Injected with an equivalent amount of saline.
[c] Mean ± SE.
[d] Significantly different from control $P < 0.001$.

from human monocytes were concentrated and fractionated on Sephadex G-50 (Table II). Approximately four million monocytes yielded enough crude pyrogenic supernatant to cause 1° fever in a rabbit. Less than half of the total pyrogenic activity applied to the column was recovered and most of this was associated with the lower molecular weight eluant.

A comparison of the LEM activities of the crude supernatant and the two different Sephadex fractions is shown in Table III. When equal pyrogen doses (as predetermined in rabbits) were injected into rats, the crude supernatant and the two Sephadex fractions gave similar lowering of plasma iron and zinc and approximately the same increases in blood neutrophils.

Discussion. In agreement with previous investigations (7), a 38,000 mol wt pyrogen was produced by human blood monocytes. Most of the activity, however, was found in the 15,000 mol wt fraction which corresponds to the pyrogen produced by neutrophils (7). Since there was too much of this activity for it to be attributable to the contaminating neutrophils (7, 10), it seems likely that the monocyte was producing this lower molecular weight pyrogen.

Dinarello (personal communication) also is now finding the low as well as the higher molecular weight pyrogen from relatively pure preparations of human monocytes. Further, he has evidence indicating that the higher molecular weight pyrogen is an unstable trimer of the lower molecular weight pyrogen which is apparently formed under slightly alkaline conditions.

The average amount of supernatant material required to give 1° fever was derived from 3.6×10^6 monocytes which is within the range previously observed (6, 7, 10). When this dose of crude monocyte pyrogen was used, it produced changes in plasma iron, plasma zinc, and blood neutrophils similar to those previously found with a similar dose of rabbit granulocyte pyrogen (1). This was also true for the two Sephadex fractions of different molecular weight (Table III). Equivalent doses of both the high molecular weight (38,000) and low molecular weight (15,000) pyrogens produced similar changes in plasma iron, plasma zinc, and peripheral blood neutrophils. All of the endogenous pyrogens that have been investigated when injected at a comparable fever dose give similar effects when LEM activities are measured (1, 3, 11).

There is some evidence for limited species specificity for both LEM (12, 13) and leukocytic pyrogen (8, 14). The material from humans, however, seems to be quite active in both the rat and rabbit.

The 38,000 mol wt pyrogen from human monocytes is known to be quite labile (7), so some of this activity may have been lost during concentration of the supernatant or

TABLE II. PYROGEN ACTIVITY AND RECOVERY OF ACTIVITY FROM THE SEPHADEX G-50 COLUMN.

	Preparation				
	A	B	C	D	Avg
Monocytes required to give 1° fever in crude supernatant $\times 10^{-6}$	3.8	4.0	4.7	1.8	3.6
% recovery of fever activity from the column	33	24	51	56	41
% recovered activity found in 38,000 pool	9	8	23	14	13
% recovered activity found in 15,000 pool	91	92	77	86	87

TABLE III. CHANGES INDUCED IN RATS BY INJECTING EITHER THE CRUDE SUPERNATANT OR THE TWO DIFFERENT POOLS FROM THE SEPHADEX G-50 COLUMN.

Preparation injected[a]	No. rats	Δ Plasma iron μg/100 ml	Δ Plasma zinc μg/100 ml	Δ Neutrophils No./mm^3 $\times 10^{-2}$
Crude supernatant	15	117 ± 7[b]	33 ± 4[b]	40 ± 3[b]
38,000 pool[c]	12	87 ± 9	34 ± 5	37 ± 4
15,000 pool[c]	12	98 ± 7	44 ± 3	47 ± 6
Control rats	20	227 ± 8[d]	116 ± 3[d]	12 ± 1[d]

[a] The dose injected was sufficient to cause a 1° fever in rabbits.
[b] Mean ± SE.
[c] These pools are described in Methods.
[d] Actual values instead of amount of change.

on the column. Care was taken to assay this material immediately after its emergence from the column, so losses due to storage would be minimal.

Summary. Stimulated human monocytes produce two physically distinct pyrogens. Both of these partially purified pyrogens will lower plasma iron and zinc and increase blood neutrophils when injected into rats or rabbits. When equivalent pyrogenic doses were injected, the two pyrogens gave quantitatively similar leukocytic endogenous mediator activities (i.e., granulocytosis and lowering of plasma iron and zinc). These results, therefore, provide evidence that leukocytic endogenous mediator can be produced by monocytes as well as by granulocytes.

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