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Comparison of Leukocytic Pyrogen and Leukocytic Endogenous Mediator (39642)

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Recent studies (1–3) have demonstrated a variety of biological effects after injecting a crude leukocyte supernatant. Among these effects are lowered plasma iron and plasma zinc, elevated fibrinogen, fever, and an increased number of peripheral blood neutrophils. The causative agent for these effects was designated leukocytic endogenous mediator, LEM (4). Further characterization of LEM revealed many similarities to the leukocytic pyrogen molecule (1, 5) which was known to be present in the crude material. In addition to causing fever, both molecules are proteins, have similar molecular weights (6), and require a free sulfhydryl group for activity. These similarities led us to suspect that LEM and leukocytic pyrogen were the same molecule.

Murphy *et al.* (7, 8) have reported a purification scheme for rabbit leukocytic pyrogen which yields a homogenous product of very high specific activity. We have utilized Murphy's methods with slight modification for preparing and purifying leukocytic pyrogen. Throughout the procedure we tested to see if the LEM activity would be lost as the leukocytic pyrogen became more pure. Only a small amount of highly active protein can be recovered from a large volume of crude material (8); our efforts were, therefore, concentrated on comparisons of biological activities rather than on proving homogenity of the final product.

Materials and methods. Animals. Holtzman-derived rats weighing 180–300 g were maintained at 23° with 12 hr of light and 12 hr of darkness. They were fed Rockland mouse and rat diet and water *ad libitum.* New Zealand white rabbits weighing 2–4 kg were used for temperature assay.

Biological activity. Heparinized plasma was obtained via cardiac puncture 8 hr after ip injection of the test substance. Plasma iron and zinc were determined in deproteinized samples by atomic absorption spectroscopy on a Model 403 Perkin-Elmer spectrophotometer. The total blood neutrophils were determined by diluting blood with Turk's diluting fluid and counting total leukocytes in a hemocytometer, followed by a 100-cell differential count of a smear stained with Wright's stain. Fibrinogen determinations were made 24 hr after injection of the test material using a heat turbidity method (9). Protein determinations were made by the method of Lowry *et al.* (10).

Preparation and purification of leukocytic pyrogen. Each batch of leukocytic pyrogen was prepared from the peritoneal granulocytes of 20 rabbits using the methods and special precautions of Kaiser and Wood (11). This material was then purified by the methods of Murphy *et al.* (7, 8) with two modifications. The first modification was that the crude material was extracted with butanol and methanol (12) and stored as a lyophilized powder instead of in a liquid state. The second modification was the use of polyacrylamide isoelectric focusing instead of sucrose gradient isoelectric focusing. The isoelectric focusing was performed at 4° in a water-cooled electrophoresis apparatus (Hoefer Scientific Instruments, Model EF301). The gels were prepared according to the method of Wrigley as described by Haglund (13). Carrier ampholines, pH range of 3 to 10, were obtained from LKB Produkter AB, Bromma, Sweden. The leukocytic pyrogen was incorporated into the gel and focused for a minimum of 10 hr. The gels were then sliced into 2-mm sections, and each section was eluted overnight in acetate buffer containing 0.1% mercaptoethanol. The eluate was dialyzed against Tris-buffered saline for 2 hr before injection.

After each purification step the leukocytic pyrogen was tested for pyrogenic activity in rabbits and for plasma iron, plasma zinc, neutrophil, and fibrinogen activity (LEM

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activity) in rats. In addition, each fraction from the various columns was tested for all activities before pooling.

Results. The yields and specific activities of a typical purification of leukocytic pyrogen at the various stages are presented in Table I. Yields, after the Sephadex CM step for five separate preparations, ranged from 4 to 31%. The percentage of yield was based on the total units of fever-producing activity recovered. After each step of the purification, an aliquot of the pooled material was assayed at three different doses, and a log dose–response curve was fitted. The specific activities increased dramatically through the first three steps but, after the fourth step, the protein concentration was too low to measure.

Throughout the purification, each fraction from the respective columns was assayed separately to avoid possible loss of resolution due to pooling of samples. In each case, the fractions containing leukocytic pyrogen activity also contained LEM activity. Conversely, the fractions that did not show leukocytic pyrogen activity did not contain LEM activity. It was also noted that the maximum response for each activity was found in the same fraction. As an example, Fig. 1 shows the responses to the different slices of a polyacrylamide isoelectric focusing gel. All five activities were found in the same area of the gel, with over 70% of the activity in two adjacent 2-mm slices. The pH in this area of the gel ranged from 7.7 to 7.9.

Table II compares the changes produced by the amount of leukocytic pyrogen necessary to cause 1° fever after various stages of purification. To make these comparisons, we determined from a three-point log dose–response curve the dose necessary to give 1° fever and, then, determined the change in the other activities at this dose. No significant changes were observed in the ratios of the various activities during purification. Most of the variation was less than that expected from a doubling of the dose or the

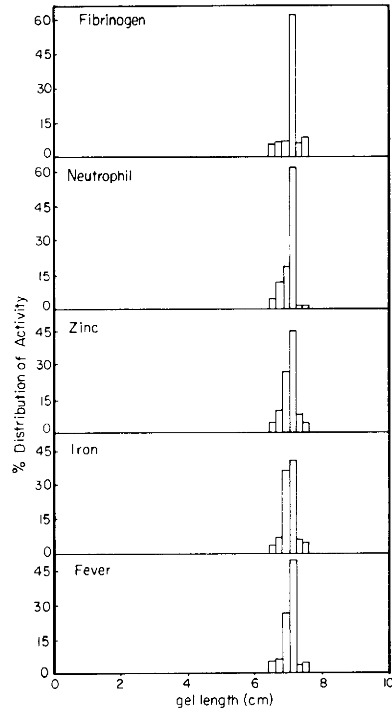

FIG. 1. Effects of polyacrylamide gel isoelectric focusing on the relative distribution of the various biological activities of leukocytic endogenous mediator. Each bar represents the mean response of six animals and is the average from two isoelectric focusing runs. The following changes are equal to 100% activity: fever, 1.56°; plasma iron lowering, 130 μg%; plasma zinc lowering, 89 μg%; increased neutrophil count, 5700/mm³; increased fibrinogen concentration, 158 mg%.

TABLE I. YIELD OF LEM AT VARIOUS STAGES OF PURIFICATION.

Purification step	Protein concentration (mg/ml)	Fever (total units)	Specific activity (units of fever/mg of protein)	Yield (%)
Crude	2.58	2350	2.3	100
Solvent extracted and filtered	0.18	1818	25.2	77
Sephadex G-50	0.01	600	227	25
Sephadex QAE	<0.01[a]	359	–	15
Sephadex CM	<0.01[a]	273	–	12
Isoelectric focusing gel	<0.01[a]	182	–	8

[a] Less than 10 μg/ml as measured by the method of Lowry *et al.* (10).

TABLE II. Changes Produced by the Amount of LEM Necessary to Cause 1° Fever.

Purification step	n[a]	Δ Plasma iron (μ/100 ml)	Δ Plasma zinc (μg/100 ml)	Δ Neutrophils (per mm³)	Δ Fibrinogen (mg/100 ml)
Crude	29	96 ± 6[b]	55 ± 4	3753 ± 388	96 ± 7
Solvent extracted and filtered	18	90 ± 5	49 ± 5	4654 ± 671	ND[c]
Sephadex G-50 purified	32	94 ± 6	64 ± 6	4225 ± 534	ND
Sephadex QAE purified	18	95 ± 9	48 ± 6	4580 ± 688	ND
Sephadex CM purified	24	98 ± 7	48 ± 4	4450 ± 600	ND
Isoelectric focusing gel	12	103 ± 12	67 ± 6	3875 ± 490	108 ± 9

[a] Number of animals assayed.
[b] Mean ± SE.
[c] Not determined, ND.

normal biological variation. In the dose-sensitive region of the log dose–response curve, a doubling of the dose caused the following changes: fever, 0.27°; plasma iron, 25 μg%; plasma zinc, 16 μg%; neutrophils, 750/mm³; and fibrinogen, 31 mg%.

Discussion. This purification of leukocytic pyrogen compares favorably with that of Murphy *et al.* (8) through the first three steps where specific activities can be determined. Minor modifications were made in the procedure which should lead to a more homogenous product. The extraction with butanol and methanol was added to remove lipids and other unidentified materials that would interfere with filtration and concentration. This step also stabilizes the crude material; hence, it can be stored without loss of activity. The only other alteration of the procedure was to use polyacrylamide-stabilized gels for isoelectric focusing. No difficulty was experienced with precipitation of proteins during the isoelectric focusing, in contrast to the work of Murphy *et al.* (7) in which urea and dimethylformamide were necessary to prevent precipitation in the sucrose-stabilized system. The isoelectric point of the active material appeared to be in the pH range of 7.7 to 7.9, in agreement with the published value of 7.78 (7). This was not a preparative scale system but it served our purposes adequately, since we began with approximately one-twentieth the amount used by Murphy *et al.* (7) and a larger percentage of the material was consumed by the additional assays before the isoelectric focusing step.

A major concern when purifying a leukocytic pyrogen is to prevent contamination by bacterial endotoxin. In addition to following procedures that minimize this possibility (8, 11), several observations were made indicating that the material purified was not an endotoxin. The active material eluted from the gel filtration columns had a much lower molecular weight than endotoxin and unlike endotoxin passed through an Amicon XM-50 filter. LEM was very labile and lost activity under the relatively mild conditions of isoelectric focusing unless a reducing agent was present. In addition, previous studies in this laboratory have shown that these effects were not due to endotoxin contamination (14–16). Other investigators have also published evidence that LEM is not endotoxin (17).

Five separate batches of leukocytic pyrogen have been purified and tested via this same procedure. In each case, the leukocytic pyrogen and LEM activities migrated as a single unit at every stage of the purification procedure. It appears unlikely that the two proteins would be so closely related that they would have the same molecular weight (6), the same affinities for positively and negatively charged ion-exchange columns, the same migration on disc gel electrophoresis (1), the same isoelectric point, and the same requirement of a free sulfhydryl group for activity (1). It appears that all the effects studied, the changes in body temperature, plasma iron and zinc levels, the number of peripheral blood neutrophils, and the fibrinogen levels are all triggered by a single protein. It would, perhaps, be prudent to withhold final judgment on the possibility of a single molecule being responsible for all of these biological activities until a check can be made with a protein of proven homogenity (8). These results, however, do suggest

strongly that leukocytic pyrogen has many biological activities in addition to inducing fever. We prefer the name LEM for this interesting protein, since it indicates the multiple activities of this mediator.

LEM seems to be a common mediator for numerous acute phase changes which normally accompany infection or inflammation. This information should aid in unraveling this important sequence of events and assist in determining their roles in host protection (18).

Summary. Leukocytic pyrogen was subjected to a five-step purification scheme, and quantitative assays for both leukocytic pyrogen and leukocytic endogenous mediator (LEM) were made. At each step of the purification scheme, the active fractions, in addition to producing fever, lowered plasma iron and zinc concentrations, elevated fibrinogen concentrations, and caused a release of neutrophils from bone marrow. It appears, therefore, that LEM and leukocytic pyrogen are the same molecule.

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