Sources of Leukocytic Endogenous Mediator in the Rat (37703)

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Previous studies showed that a variety of alterations in host metabolism can be induced by proteins released from properly stimulated polymorphonuclear leukocytes (1–7). Upon injection into rats, leukocytic endogenous mediator (LEM) will lower plasma iron (1, 3) and zinc (2, 3), increase serum copper (7) and acute phase globulin (4), cause a shift of amino acids from muscle to liver (6), and release neutrophils from bone marrow (5).

The methods which have been used for the preparation of LEM are the same as those used for endogenous pyrogen. The name endogenous mediator has been used to indicate that a wider range of biological activities is involved and to suggest that different proteins may be responsible. The evidence, to date, suggests that all of these biological activities are caused by the same or a closely related group of proteins (8).

Endogenous pyrogen can be synthesized by rabbit peripheral blood leukocytes, macrophages, and Kupffer cells *in vitro* after proper stimulation in a complete tissue culture media (9). The other biological activities attributed to LEM have not been investigated with cells from these sources. One difficulty in such an investigation is that studies on LEM have been conducted in rats, whereas the rabbit is generally used in experiments on endogenous pyrogen. The rat, unlike the rabbit, will not respond with a fever to endotoxin (10) or endogenous pyrogen (11).

The source of the leukocytes producing LEM has usually been peritoneal exudates of the rabbit or rat (1–7). Active preparations from the monkey and dog also have been obtained (12). Some evidence has indicated that peripheral blood leukocytes can be stimulated *in vivo* (3, 13, 14). The apparent formation of a heat labile endogenous factor after injection of endotoxin in the rat (13) was later attributed to the formation of a protein–endotoxin complex (15). Pekarek and Beisel (3) induced a heat labile, serum endogenous factor in rats with materials that did not contain endotoxin. It has not, however, been clearly established that this serum activity was entirely endogenously derived.

It will be the purpose of this investigation to see if methods of stimulation used for synthesis of endogenous pyrogen in rabbit cells will also cause these various types of cells from rats to produce LEM. A further purpose will be to provide better evidence that the serum activity after injection of *Diplococcus pneumoniae* in rats is due to LEM.

Materials and Methods. Animals. All of the activities were measured in female Holtzman rats weighing 180–200 g. They were fed Rockland rat diet and water *ad libitum*. The animal rooms were routinely maintained at 72° F with 12 hr of light and 12 hr of darkness. Retired female breeders, also from Holtzman, were used as a source for peripheral blood leukocytes, Kupffer cells, and peritoneal macrophages.

Preparation of LEM from rat peritoneal granulocyte exudates. Rats were infused ip with 50 ml of 0.2% shellfish glycogen in saline. Sixteen hours later 3×10^9 heat-killed *Staphylococcus aureus* in saline were injected. Two hours after this stimulation the exudates were removed and incubated under conditions previously described (8). The volume of supernatant was adjusted to 1×10^8 leukocytes/ml in the incubation mixture and the results expressed as the activity obtained when the supernatant from a given number of leukocytes was injected into each rat.

Isolation of peritoneal macrophages, peripheral blood leukocytes, and liver Kupffer cells. Peritoneal exudates containing approximately 85% macrophages were obtained 3 days after injecting rats with 30 ml of sterile thioglycollate medium (16). In some experiments, as indicated in the text, macrophages were collected 5 days after ip injection of 10 ml of mineral oil (17). Peripheral blood leukocytes were isolated using 3% dextran (mol wt 252,000) in saline (18). The precautions used by Bodel and Atkins (19) were taken in these experiments. Kupffer cells were isolated by forcing small chunks of liver through a 30-mesh stainless steel wire cloth in the barrel of a syringe. This liver suspension was stirred in the cold with 3% EDTA in saline, and the buffy coat separated by centrifugation (20).

Stimulation of leukocytes or Kupffer cells in vitro. After isolation the cells were washed with the incubation media. The complete media used were McCoy's 5A or Eagle's minimum essential medium with 10% rat serum. In some experiments Hank's balanced salt solution or physiological saline was used. A number of different stimuli were tried with approximately the same results. The only one to be reported in the accompanying tables will be heat-killed *Staphylococcus aureus* (ATC 13710). These cells were from an 18-hr broth culture and were suspended in saline and autoclaved for 15 min at 120° (19). Approximately 15 of these bacteria were added for each phagocytic cell in the culture. Supernatants from heat-killed *S. aureus* were found inactive for lowering plasma iron or increasing neutrophils and were nonpyrogenic (19).

Stimulation of macrophages in vivo. Macrophages were produced in the peritoneal cavity by injecting 30 ml of thioglycollate medium. Three days later, each rat was injected with 3×10^9 heat-killed *S. aureus* in 50 ml sterile saline. Two hours after injecting the bacteria, the macrophages were isolated and incubated for 16 hr in saline containing antibiotics and heparin (8).

Production and testing of serum activity after injecting D. pneumoniae. *D. pneumoniae,* type 5-A, was obtained from Dr. Robert Pekarek. Serum activity was produced by modifications of the methods of Pekarek and Beisel (3). Blood was taken 4 hr after injecting ip 1×10^{10} viable *D. pneumoniae.* After the blood was allowed to clot, the serum was separated and filtered first through a 1.2-μm and then a 0.22-μm Millipore filter. Plasma was checked for LEM activity 8 hr after the last of a series of 6 daily injections of 1 ml of the filtered serum.

Avoiding bacterial or endotoxin contamination. A portion of each culture was incubated in nutrient broth, and any which contained bacteria were discarded. The usual precautions were taken in using glassware and solutions free of endotoxin (11). A control sample, containing the same phagocytic cells and media but not the stimulator, was used in each experiment. For further assurance that the activities were not due to endotoxin, periodic tests were made which would distinguish between effects due to LEM and endotoxin (8).

Measuring biological activities. Plasma iron concentration (1), plasma zinc concentration (2), and total blood neutrophils (5) were measured in rats 8 hr after an ip injection of the test dose, and these methods have been described previously (8).

Results. Before using any of these methods on rat cells, attempts were made to duplicate previous studies on the activation of rabbit peripheral blood leukocytes. The cells were incubated in McCoy's 5A medium with 10% rabbit serum and stimulated by adding heat-killed *S. aureus*. The supernatant from 5×10^7 total rabbit blood leukocytes produced an average increase in temperature of 1.5° when injected into normal rabbits. When this same dose was used on each of a group of 10 rats, plasma iron was lowered to 112 ± 7 μg/100 ml and neutrophils increased to $6350 \pm 360/mm^3$. Control rats receiving supernatant material from cells incubated without *S. aureus* had plasma iron concentrations of 249 ± 9, and peripheral blood neutrophils averaged 1650 ± 290.

Attempts to produce LEM *in vitro* from macrophages, Kupffer cells, or peripheral blood leukocytes from rats are shown in Table I. The supernatant from macrophages incubated with *S. aureus* upon injection into normal rats lowered plasma iron and in-

TABLE I. Production of LEM by Various Tissues *in Vitro*.

Tissue	No. of cells[a]	Incubation media[b]	Stimulus[c]	No. trials	Plasma iron (μg/100 ml)	Neutrophils (no. $\times 10^{-2}$ /mm^3)
None	None	None	None	46	255 ± 8[d]	12 ± 1[d]
Macrophages	1×10^8	McCoy 5A	None	13	235 ± 19	15 ± 2
Macrophages	1×10^8	McCoy 5A	*S. aureus*	13	182 ± 21[e]	29 ± 3[e]
Kupffer cells	5×10^7	Eagle's MEM	None	14	234 ± 14	23 ± 4
Kupffer cells	5×10^7	Eagle's MEM	*S. aureus*	20	206 ± 17	39 ± 4[e]
Blood leukocytes	1×10^8	McCoy 5A	None	8	265 ± 33	18 ± 3
Blood leukocytes	1×10^8	McCoy 5A	*S. aureus*	8	215 ± 31	33 ± 4[e]
Blood leukocytes	1×10^8	Hanks	None	12	244 ± 13	17 ± 4
Blood leukocytes	1×10^8	Hanks	*S. aureus*	29	186 ± 9[e]	33 ± 4[e]

[a] Number of cells used to produce the supernatant which was injected into each test rat.
[b] Each incubation media contained 10% rat serum. MEM, minimum essential medium.
[c] Approximately 15 bacteria were added for each phagocytic cell. The bacteria and phagocytic cells were removed by centrifugation before injecting the supernatant.
[d] Mean $\pm$ standard error.
[e] Significantly different with $p < 0.05$ from the trials without *S. aureus*.

creased peripheral blood neutrophils. The amount of activity was low, so that it might be due to the LEM produced by the approximately 10% granulocytes contaminating the peritoneal exudate. We were unable to increase this activity by using mineral oil rather than thioglycollate to produce the exudates. Changing the incubation media did not alter the results. A balanced salt solution produced just as much activity as either Eagle's or McCoy's tissue culture media.

The use of liver slices rather than isolated Kupffer cells produced supernatants which would lower plasma iron and increase peripheral blood neutrophils, but the amount of activity was low. Rat blood leukocytes, unlike the rabbit cells, gave just as much activity with a balanced salt solution as with a complete tissue culture media.

Macrophages were stimulated *in vivo* by giving an ip injection of 3×10^9 *S. aureus* 3 days after thioglycollate. The activities of supernatants from macrophages were compared to those obtained from peritoneal granulocytes (Table II). When extracts from

TABLE II. Comparison of LEM Activity from Peritoneal Macrophages and Granulocytes Stimulated *in Vivo* with *S. aureus*.[a]

Determination	Cell source	No. of cells used to prepare each dose				
		Control[b]	1×10^7	5×10^7	1×10^8	5×10^8
Plasma iron (μg/100 ml)	Granulocytes	283 ± 18[c]	199 ± 16	135 ± 9	116 ± 7	82 ± 6
	Macrophages	274 ± 15	260 ± 14	160 ± 17	135 ± 11	94 ± 4
Plasma zinc (μg/100 ml)	Granulocytes	125 ± 5	103 ± 6	81 ± 6	72 ± 5	46 ± 4
	Macrophages	122 ± 5	107 ± 8	60 ± 7	58 ± 6	39 ± 6
Blood neutrophils (no. $\times 10^{-2}$/mm^3)	Granulocytes	14 ± 2	30 ± 3	44 ± 2	46 ± 2	60 ± 3
	Macrophages	16 ± 2	20 ± 2	39 ± 3	49 ± 3	62 ± 3

[a] Granulocytes were produced by infusing glycogen and macrophages by thioglycollate (see Methods). Two hours before harvesting the cells, 3×10^9 heat-killed *Staphylococcus aureus* were injected ip.
[b] Supernatant from 1×10^8 cells without *S. aureus*.
[c] Mean $\pm$ standard error. Each group contained 6–12 rats.

TABLE III. Demonstration of LEM in Serum Four Hours After Injecting *D. pneumoniae* and Effects of Repeated Injections of 4-hr Serum.

Recipient rat	Amount of 4 hr serum injected ip	No. trials	Plasma iron (μg/100 ml)	Blood neutrophils (no. $\times$ 10^{-2}/mm^3)
Normal	Control[a]	14	241 ± 7[b]	14 ± 2[b]
Normal	1 ml	20	112 ± 7[c]	68 ± 6[c]
Normal	6 daily of 1 ml	11	142 ± 7[c]	64 ± 7[c]
Tolerant to 1 μg endotoxin[d]	1 ml	6	123 ± 11[c]	55 ± 5[c]

[a] One milliliter of normal serum. Plasma iron and peripheral blood neutrophils 8 hr later.
[b] Mean ± standard error.
[c] Significantly different from control, $p < 0.01$.
[d] One microgram of *E. coli* endotoxin daily for 6 days (9).

1×10^8 macrophages were injected into normal rats, it caused a 50% decrease in plasma iron and plasma zinc concentrations and a threefold increase in the peripheral blood neutrophils. These changes were too large to be attributed to contamination with granulocytes.

Table III shows the effects of repeated daily injections of 4-hr serum from rats injected with *D. pneumoniae*. There was very little indication of the development of tolerance. The 4-hr serum also was active in rats that had been made tolerant to 1 μg of endotoxin. Still other evidence that the serum activity was due to LEM and not bacterial or other contamination was indicated by fever production with a short latency in endotoxin refractory rabbits and a threefold increase in peripheral blood neutrophils 1 hr after injection of the 4-hr serum. Neither endotoxin nor *D. pneumoniae* produced significant elevations in neutrophils at 1 hr.

Discussion. Supernatants prepared by incubating peritoneal granulocytes from the rat and rabbit yield approximately equal LEM activities when tested in the rat (1). However, only the rabbit material gives a pyrogenic response (11). In checking for possible new cell sources for LEM production, it seemed advisable to use the same species for obtaining the cells and checking activity even though fever could not be measured. The preliminary experiments showing endogenous pyrogen synthesis by *in vitro* incubation of rabbit peripheral blood leukocytes are in agreement with previous investigations (9). When this material containing endogenous pyrogen was injected into rats, it also gave LEM activity, *i.e.,* decreased plasma iron and increased peripheral blood neutrophils. Thus, further evidence was provided for the similarity and possible identity of LEM and endogenous pyrogen (8).

Macrophages stimulated with *S. aureus* in the peritoneal cavity of rats yield as much LEM as peritoneal granulocytes. Attempts at producing LEM *in vitro* from rat macrophages with the same stimuli gave low yields. There was some indication that a complete tissue culture media was no better than a balanced salt solution. Rabbit Kupffer cells, mononuclear, or normal blood leukocytes require specific stimulus and a fully supportive media for endogenous pyrogen production *in vitro* (9, 20). Rabbit peritoneal granulocyte exudates will produce LEM or endogenous pyrogen without any previous specific stimuli (8, 9). Although in our early studies (1) we obtained LEM from rat peritoneal granulocytes without additional stimulation, we have been unable to repeat this in more recent experiments. Both granulocytes and macrophages from rat peritoneal exudates are inactive unless first stimulated *in vivo* (Table II). It appears, therefore, that the proper *in vitro* incubation conditions for stimulation of rabbit and rat cells are different and that the ideal conditions for rat cells have not been attained.

In the experiments on LEM activity in serum after injecting *D. pneumoniae,* a higher dose was necessary than had been used previously by Pekarek and Beisel (3). In agreement with their results we found a serum activity for lowering plasma iron which could be destroyed by heating at 90° for 30 min.

Rat serum obtained 4 hr after injecting *D. pneumoniae* also increased the peripheral blood neutrophils of normal rats threefold within 1 hr after its injection. Further evidence for LEM in the 4-hr serum was the failure to produce tolerance and retaining activity in endotoxin-tolerant rats. We therefore concur with Pekarek and Beisel (3) on a serum endogenous mediator after injecting *D. pneumoniae* and believe that such a mediator after injecting endotoxin must still remain in doubt (15).

Summary. After stimulation *in vivo* with heat-killed *Staphylococcus aureus,* rat peritoneal granulocytes and macrophages produced leukocytic endogenous mediator (LEM). When this LEM was injected into normal rats, it lowered plasma iron and zinc concentrations and markedly increased blood neutrophils. Incubation of macrophages, blood leukocytes, or Kupffer cells with *S. aureus* in tissue culture media and rat plasma *in vitro* gave low yields of LEM. Further evidence was presented to show that serum taken from rats 4 hr after an ip injection of *Diplococcus pneumoniae* contained LEM.

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