Investigations on the Mode of Action of Endogenous Mediator (37303)

RALPH F. KAMPSCHMIDT, HERBERT F. UPCHURCH, AND LARRY A. PULLIAM
Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma 73401

The injection of leukocytic endogenous mediator (LEM) in rats has been shown to lower plasma iron (1, 2) and zinc concentration (3, 4), increase plasma α_2-acute phase globulin (5), cause the release of marrow neutrophils (6) and flux of amino acids from serum to liver (7). Endogenous mediator prepared from rat or rabbit peritoneal leukocytes by methods similar to those used for endogenous pyrogen, like pyrogen, was a heat-labile, low molecular weight protein which was fast acting and would not induce tolerance (1, 2, 6, 8).

The close relationship between these rather diverse biological activities suggests that they may have some common site or mode of action. One such possibility would be through either the adrenal or pituitary hormones. Endogenous mediator will also cause fever in rabbits and a hypothermia in rats (9). Temperature regulation seems to occur through the anterior portion of the hypothalamus, and chemically induced alterations in the concentration of catecholamines can profoundly modify pyrogen fever (10). In this investigation the activity of LEM was compared in normal, adrenalectomized, and hypophysectomized rats and in rats with drug altered levels of the various catecholamines.

Methods. Experimental animals and operations. Female Holtzman rats weighing 180–200 g were fed Rockland rat diet and water *ad libitum*. They were routinely maintained at 72°F with 12 hr of light and 12 hr of darkness. The hypophysectomized rats were obtained from Hormone Assay Laboratories, Chicago, Illinois. Bilateral adrenalectomy was performed under ether anesthesia and autopsy was used at the end of the experiment to assure complete removal of adrenal tissue. Following adrenalectomy the rats were given a 0.9% solution of sodium chloride to drink.

Preparation of endogenous mediator. Endogenous mediator was prepared from rabbit peritoneal leukocytes by the methods used for endogenous pyrogen (11). Partial purification was achieved by the modified butanol–methanol method described by Rafter *et al.* (12). Special precautions used to avoid bacterial or endotoxin contamination have been previously described (1, 4). The doses employed are expressed in terms of the number of leukocytes in the original preparation.

Biological activity. Changes measured in rats after injecting LEM or various drugs were plasma iron concentration and the number of neutrophils in the peripheral blood. Methods used in determining these have been previously described (1, 6). These measurements were made 8 hr after injecting LEM, catecholamines, or various drugs employed to stimulate or inhibit catecholamines.

Drugs and methods of administration. All intracisternal (ic) injections were administered with a Hamilton microliter syringe and a 27-gauge needle using a total volume of 0.01 ml in rats lightly anesthetized with ether (13). L-Adrenaline bitartrate, L-noradrenaline, and 5-hydroxytryptamine creatine sulfate (K and K Laboratories) were dissolved in saline and injected ic.

Reserpine base (K and K Laboratories) was dissolved in glacial acetic acid, diluted with 0.3 *M* sucrose and injected iv (14). The method of Breese and Traylor (15) was used to deplete dopamine and norepinephrine. Two ic injections of 6-hydroxydopamine (200 μg/rat) dissolved in 0.9% NaCl containing 1 mg/ml of ascorbic acid were given 7 days apart.

p-Chlorophenylalanine (Sigma Chemical Co.) was dissolved in 0.5 ml 0.9% NaCl con-

TABLE I. Effects of Adrenalectomy or Hypophysectomy on the Activity of LEM.

	Controls			LEM[a]		
	No. trials	Plasma iron (μg/100 ml)	Blood neutrophils (no./mm^3)	No. trials	Plasma iron (μg/100 ml)	Blood neutrophils (no./mm^3)
Normal rats	18	187 ± 13[b]	1239 ± 153[b]	15	66 ± 5[b]	5352 ± 720[b]
Sham adrenalectomy	10	171 ± 17	1584 ± 189[c]	10	69 ± 4	6458 ± 610
Adrenalectomy	12	111 ± 7[c]	1690 ± 262[c]	18	43 ± 4[c]	5319 ± 525
Hypophysectomy	10	113 ± 9[c]	1107 ± 106	27	67 ± 5	5792 ± 579

[a] Partially purified LEM prepared from 1 × 10^8 rabbit peritoneal leukocytes was injected ip into each rat 8 hr before these determinations.

[b] Mean ± SE.

[c] Signficantly different from normal: $p < 0.01$.

taining 3 drops 4 *N* NaOH and then neutralized with 1 *N* HCl and injected ip (16). 5-Hydroxytryptophan was suspended in water at a concentration of 7.5 mg/ml and injected ip (17). Iproniazid, a monoamine oxidase inhibitor (Aldrich Chemical Co.), was dissolved in 0.9% NaCl and injected ip.

Results. The effect of adrenalectomy and hypophysectomy on the activity of LEM in rats is shown in Table I. Plasma iron concentration was lowered by both adrenalectomy and hypophysectomy. The number of neutrophils in the peripheral blood seemed to be increased slightly by the operation to remove the adrenals. LEM was equally active in lowering plasma iron concentration and in releasing marrow neutrophils in normal, adrenalectomized, and hypophysectomized rats.

A comparison of ic and iv injection of LEM at various doses is presented in Table II. There was a progressive decrease in plasma iron concentration and an increase in blood neutrophils with increasing iv doses of LEM. The ic injections were less active than the iv at low doses of LEM, but much more active than the iv when high doses were used.

The failure in ic injections of LEM to give definitive results led to further studies of the possible involvement of the hypothalamus and catecholamines. The effects of ic injection of adrenaline, noradrenaline, or serotonin on plasma iron concentration and release of marrow neutrophils are shown in Table III. With the possible exception of adrenaline, these catecholamines had very little effect on either plasma iron concentration or the number of blood neutrophils at 8 hr after injec-

TABLE II. A Comparison of ic and iv Injections of LEM at Various Doses.

Amount LEM injected[a]	Intravenous		Intracisternal	
	Plasma iron (μg/100 ml)	Neutrophils (no./mm^3)	Plasma iron (μg/100 ml)	Neutrophils (no./mm^3)
Saline only	252 ± 10[b]	1384 ± 154[b]	251 ± 11[b]	1357 ± 138[b]
1 × 10^5	223 ± 13[c]	1731 ± 171	279 ± 16[c]	1607 ± 157
1 × 10^6	188 ± 11[c]	1781 ± 183[c]	237 ± 10	1833 ± 179[c]
1 × 10^7	155 ± 12[c]	2818 ± 331[c]	134 ± 9[c]	4556 ± 461[c]
1 × 10^8	123 ± 9[c]	5202 ± 528[c]	56 ± 6[c]	6571 ± 672[c]
Control	247 ± 15	1121 ± 64	247 ± 15	1121 ± 64

[a] Each dose of partially purified LEM was injected in a total volume of 0.01 ml (see Methods).

[b] Mean ± SE for groups of 12–18 rats.

[c] Significantly different from saline control: $p < 0.01$.

TABLE III. Effect of ic Injections of Various Catecholamines on Plasma Iron Concentration and Blood Neutrophils in Rats.

Catecholamine	Amount (μg/kg B.W.)	Plasma iron (μg/100 ml)	Neutrophils (no./mm³)
Normal rats	—	279 ± 21[a]	714 ± 78[a]
Saline control	—	266 ± 22	743 ± 82
Adrenaline	200	183 ± 21	2149 ± 374
Noradrenaline	300	305 ± 19	1101 ± 112
5-Hydroxytryptamine	300	258 ± 19	1047 ± 106

[a] Each value is the mean ± SE for 6 rats.

tion. It was possible that catecholamines were unable to enter the hypothalamus. Materials were therefore injected which have been shown to alter the cellular concentrations of various catecholamines.

Effects of drugs, which are known to alter the concentration of various catecholamines, on plasma iron and blood neutrophils when administered alone or in combination with LEM are shown in Table IV. Depression of 5-hydroxytryptamine and noradrenaline levels with reserpine had a slight effect on both plasma iron and blood neutrophils. It also caused enhancement of the effects produced by LEM. The use of 6-hydroxydopamine to deplete dopamine and reduce the levels of norepinephrine also gave slight effects when given alone but did not influence effects produced by LEM. Depleting the levels of serotonin with *p*-chlorophenylalanine caused a small decrease in plasma iron concentration and an increased number of blood neutrophils. Increasing the level of serotonin with 5-hydroxytryptophan also produced a decrease in plasma iron and a marked increase in the number of neutrophils.

Discussion. It was previously shown that hypophysectomy (18) and adrenalectomy (19) will produce hypoferremia in rats. Fever (20) and hypoferremia (19), however, could still be accentuated by severe stress in the absence of the adrenals. From these and the results of the present experiments it seems unlikely that LEM acts through the hormones produced by these tissues.

Endogenous pyrogen seems to produce fever by stimulus to hypothalamic thermoreceptors (10). In paired experiments, the same dose of endogenous pyrogen caused a much greater increase in body temperature

TABLE IV. Activity of LEM in Rats with Drug Altered Concentrations of Various Catecholamines.

		Drug only		Drug plus LEM[a]	
Drug injected[b]	Amount (mg/kg B.W.)	Plasma iron (μg/100 ml)	Neutrophils (no./mm³)	Plasma iron (μg/100 ml)	Neutrophils (no./mm³)
Vehicle only	—	263 ± 10[c]	1046 ± 94[c]	106 ± 8[c]	6974 ± 420[c]
Reserpine	2.5	224 ± 18[d]	1530 ± 136[d]	51 ± 6[d]	8522 ± 723[d]
6-Hydroxydopamine	1	220 ± 16[d]	2150 ± 231[d]	109 ± 12	6646 ± 484
p-Chlorophenylalanine	316	201 ± 21[d]	2511 ± 310[d]	117 ± 14	4983 ± 562
Iproniazid	100	295 ± 17	3228 ± 264[d]	—	—
5-Hydroxytryptophan	75	194 ± 15[d]	5274 ± 513[d]	—	—
Iproniazid + 5 HT	100 + 75	169 ± 16[d]	5743 ± 486[d]	—	—

[a] Partially purified LEM prepared from 1×10^8 rabbit peritoneal leukocytes was injected ip into each rat 8 hr before these determinations.

[b] For a description of the vehicle and method of administration, see Methods.

[c] Mean ± SE for groups of 6–12 rats.

[d] Significantly different from injection of vehicle, $p < 0.01$.

when given ic that when given iv (21). Although this was also true for the action of LEM on plasma iron concentration and blood neutrophils when injected in high doses, it was not true when lower doses were used. This led to further investigation of catecholamines.

Injection of catecholamines into brain produced marked changes in body temperature, but considerable variation in the response between species was observed (10). Plasma iron concentration and numbers of blood neutrophils in rats showed very little change after ic injection of various catecholamines. It is possible that these materials will not reach the hypothalamus in sufficient concentration when injected ic. It has been shown, however, that various catecholamine levels were altered in rats by giving drugs at doses shown in Table IV (13–16, 22). Administration of 5-hydroxytryptophan and iproniazid produced a marked increase in the number of peripheral blood neutrophils. This might be due to increased amounts of serotonin in any of a number of different tissues (17). Lowering serotonin by injecting reserpine (14) or *p*-chlorophenylalanine (16) however, failed to alter effects produced by LEM on blood neutrophils. Therefore, there was very little evidence that LEM acted by alteration of levels of catecholamines in various tissues.

It would appear unlikely that the diverse biological changes produced by LEM could be due to a single protein or a common mechanism of action, since their primary sites of action would be expected to be quite different (23, 24). Attempts to separate proteins responsible for these biological changes produced by LEM have been unsuccessful (8). The crude LEM supernatant from rabbit peritoneal leukocytes was purified about 300-fold with approximately equal retention of biological activity for fever, lowering of plasma iron and zinc, releasing of bone marrow neutrophils, and increasing plasma α_2-acute phase globulin (8). In spite of these findings there remains some evidence that proteins of LEM and endogenous pyrogen may be different (9, 24). The present studies will not help resolve whether LEM and endogenous pyrogen are distinct proteins, but they do indicate that the mechanism of action for fever production and the other alterations caused by LEM are different.

Summary. An attempt was made to find some common site or mode of action for the numerous biological effects produced by leukocytic endogenous mediator (LEM). The effects of LEM on plasma iron concentration and number of blood neutrophils were compared in adrenalectomized and hypophysectomized rats by injecting intracisternally as compared to intravenously and after alteration of the concentration of catecholamines. The results obtained suggested that LEM was not acting through the adrenal or pituitary hormones or by modifying the levels of catecholamines. The results also indicated that the mechanism of fever production was different from the other changes produced by LEM.

1. Kampschmidt, R. F., and Upchurch, H. F., Amer. J. Physiol. **216**, 1287 (1969).

2. Pekarek, R. S., and Beisel, W. R., Proc. Soc. Exp. Biol. Med. **138**, 728 (1971).

3. Pekarek, R. S., and Beisel, W. R., Fed. Proc. **28**, 691 (1969).

4. Kampschmidt, R. F., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **134**, 1150 (1970).

5. Eddington, C. L., Upchurch, H. F., and Kampschmidt, R. F., Proc. Soc. Exp. Biol. Med. **139**, 565 (1972).

6. Kampschmidt, R. F., Long, R. D., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **139**, 1224 (1972).

7. Wannemacher, R. W., Jr., Pekarek, R. S., and Beisel, W. R., Proc. Soc. Exp. Biol. Med. **139**, 128 (1972).

8. Kampschmidt, R. F., Upchurch, H. F., Eddington, C. L., and Pulliam, L. A., Amer. J. Physiol. (in press).

9. Kampschmidt, R. F., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **131**, 864 (1969).

10. Snell, E. S., *in* "Microbial Toxins" (S. Kadis, G. Weinbaum, and S. J. Ajl, eds.), Vol. 5, p. 277. Academic Press, New York (1971).

11. Kaiser, H. K., and Wood, W. B., Jr., J. Exp. Med. **115**, 27 (1962).

12. Rafter, G. W., Cheuk, S. F., Krause, D. W., and Wood, W. B., Jr., J. Exp. Med. **123**, 433 (1966).

13. Schanberg, S. M., Schildkraut, J. J., and Kopin, I. J., J. Pharmacol. Exp. Ther. **157**, 311 (1967).

14. Azzaro, A. J., Wenger, G. R., Craig, C. R., and Stitzel, R. E., J. Pharmacol. Exp. Ther. **180**, 558 (1972).

15. Breese, G. R., and Traylor, T. D., J. Pharmacol. Exp. Ther. **174**, 413 (1970).

16. Kol, K., and Weissman, A., J. Pharmacol. Exp. Ther. **154**, 499 (1966).

17. Udenfriend, S., Weissbach, H., and Bogdanski, D. F., J. Biol. Chem. **224**, 803 (1957).

18. Crafts, R. C., and Walker, B. S., Endocrinology **41**, 340 (1947).

19. Hamilton, L. D., Gubler, C. J., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M., Endocrinology **48**, 44 (1951).

20. Haider, R., Proc. Soc. Exp. Biol. Med. **136**, 514 (1971).

21. Adler, R. D., and Jay, R. J. T., Proc. Soc. Exp. Biol. Med. **119**, 660 (1965).

22. Ingeito, A. J., and Bonnycastle, D. D., Can. J. Physiol. Pharmacol. **45**, 723 (1967).

23. Eddington, C. L., Upchurch, H. F., and Kampschmidt, R. F., Proc. Soc. Exp. Biol. Med. **136**, 159 (1971).

24. Pekarek, R. S., Wannemacher, R. W., Jr., Chappel, F. E., III, Powanda, M. C., and Beisel, W. R., Proc. Soc. Exp. Biol. Med. **141**, 643 (1972).

Received Jan. 30, 1973. P.S.E.B.M., 1973, Vol. 143.