Characterization of the Endogenous Mediator(s) of Serum Zinc and Iron Depression During Infection and Other Stresses (35977)

ROBERT S. PEKAREK AND WILLIAM R. BEISEL
(Introduced by Robert W. Wannemacher, Jr.)
U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Decreases in serum iron and zinc concentrations have been shown to occur during both bacterial and viral infections (1–4), endotoxemia (5–7), and a variety of other stressful conditions in man and experimental animals (5, 8, 9). However, little is known concerning the pathophysiologic mechanism leading to these alterations in zinc and iron metabolism during the inflammatory process.

In a recent series of studies, Kampschmidt *et al.* (10) and Kampschmidt and Upchurch (11) reported that the lowering of plasma iron concentrations during experimental endotoxemia in the rat may be related to the presence of an intermediate factor which appeared in the plasma within 2 hr after induced intoxication. This intermediate was a heat labile (inactivated at 90° for 30 min) substance similar in its action to endogenous pyrogen. These authors further reported (12, 13) that this endogenous pyrogen, released by polymorphonuclear (PMN) leukocytes, lowered plasma iron and zinc concentrations in the rat. Although the above authors employed only bacterial endotoxin as a stimulus, a common humoral factor, whether endogenous pyrogen or another substance or substances may be responsible for alterations in zinc and iron metabolism during the inflammatory process.

The purpose of the present investigation was to demonstrate the presence of a common endogenous mediating factor for serum zinc and iron depression in the serum of animals in response to a variety of known serum zinc and iron depressing stimuli. Attempts to partially purify and characterize this mediator are also described.

Materials and Methods. Lipopolysaccharide from *Escherichia coli* 0127:B8 (Difco Laboratories), a crystalline homopolymeric duplex of polyinosinic and polycytidylic acids (poly In·Cn) (Miles Laboratories), *Diplococcus pneumoniae,* Type 1-A, and attenuated vaccine strain of *Pasteurella tularensis,* Lot No. 4 (National Drug Company) were employed as the serum zinc and iron depressing agents. Male Dunning-Fisher rats (Microbiological Associates), weighing 175–200 g, were used in this study.

In the first series of studies, prescribed doses of the above materials were prepared and administered intraperitoneally (ip) to all donor rats as indicated in Table I. Blood samples were obtained at varying intervals from the first group of donors, as well as from saline inoculated controls, to determine the time of maximal serum zinc and iron depression and to correct for any variability associated with diurnal periodicity. The remaining infected or intoxicated donors were bled 2 hr postchallenge; and their respective serums were pooled, Millipore-filtered for sterility, and divided into two categories: untreated and heat treated (30 min at 90°) before being administered ip to normal recipient rats (Table I). Blood samples from the recipient rats were taken at 3-hr intervals to determine effect and time of effect.

In the second series of studies, crude preparations of the endogenous mediator were obtained from rat PMN leukocytes by a modified method described by Rafter *et al.* (14) for endogenous pyrogen. Rats were infused ip with a pyrogen-free normal saline

[1] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

TABLE I. Effect of 2-hr Serum of Rats Receiving Endotoxin, *D. pneumoniae*, *P. tularensis*, or Poly In·Cn on Serum Zn and Fe of Normal Recipient Rats.

Treatment of donor rats (ip)	Normal recipient rat given 2-hr serum, ip	Serum zinc (μg/100 ml)[a]	Serum iron (μg/100 ml)[a]
Saline control		145 ± 3	155 ± 7
Endotoxin, 10 μg	None	42 ± 3[b,c]	49 ± 7[b,c]
D. pneumoniae (3×10^7)	None	30 ± 4[b,c]	36 ± 8[b,c]
P. tularensis (4×10^7)	None	61 ± 4[b,c]	58 ± 7[b,c]
Poly In·Cn, 100 μg	None	81 ± 4[b,d]	88 ± 8[b,d]
Saline control		147 ± 4	159 ± 7
Endotoxin, 10 μg	Untreated, 1 ml	70 ± 4[b,d]	89 ± 8[b,d]
D. pneumoniae (3×10^7)	Untreated, 1 ml	86 ± 3[b,d]	67 ± 8[b,d]
P. tularensis (4×10^7)	Untreated, 1 ml	98 ± 3[b,d]	95 ± 7[b,d]
Poly In·Cn, 100 μg	Untreated, 1 ml	91 ± 4[b,e]	116 ± 7[b,e]
Saline control		145 ± 3	155 ± 7
Endotoxin, 10 μg	Heated,[f] 1 ml	140 ± 4	145 ± 8
D. pneumoniae (3×10^7)	Heated, 1 ml	138 ± 5	159 ± 6
P. tularensis (4×10^7)	Heated, 1 ml	140 ± 4	148 ± 8
Poly In·Cn, 100 μg	Heated, 1 ml	152 ± 3	150 ± 7

[a] Means of a minimum of 10 animals ± SE.
[b] $p < .01$, compared to controls.
[c] Time of maximum effect: 9–12 hr; [d] 6–9 hr; [e] 6 hr.
[f] Heated 30 min at 90°.

solution containing 0.2% shellfish glycogen (Mann Research Laboratories) 120,000 U/liter of penicillin G, and 0.4 g/liter of streptomycin. Peritoneal exudates were collected 12 hr after infusion, and PMN leukocytes were obtained by centrifugation. The cells were resuspended in sterile normal saline and incubated for 2 hr at 37°. The supernatant was obtained by centrifugation and represented the crude endogenous mediator. Activity was assayed by giving varying doses of the extract ip to rats and measuring the depression of serum zinc and iron concentrations at 3-hr intervals for each dose–response group and in a control group for a period of 24 hr postadministration. To exclude the possibility of serum zinc and iron reductions being produced by contaminating endotoxin, all test rats, as well as all controls used in the second series of studies, were made endotoxin tolerant by giving 10 μg of endotoxin ip daily for 7 days.

Partial purification of the endogenous mediator was attempted by employing the butanol–methanol method for endogenous pyrogen of Rafter *et al.* (14). Protein concentrations were measured by the method of Lowry *et al.* (15), and protein nitrogen was determined by Kjeldahl analysis. Estimation of the range of molecular weight (mol wt) was achieved by ultrafiltration techniques employing the Amicon model 52 ultrafiltration cell and a series of Diaflo ultrafiltration membranes with pores of varying molecular weight retention (Amicon Corporation).

Serum zinc concentrations were determined by a previously described atomic absorption spectrophotometric technique (7) and serum iron concentrations by the colorimetric method of Levy and Vitacca [cited in Ref. (16)]. Zinc and iron-free polyethylene syringes, pipettes, and test tubes were used to minimize zinc and iron contamination from exogenous sources.

Results. As shown in Table I, donor rats receiving endotoxin, virulent *D. pneumoniae*, attenuated *P. tularensis*, or poly In·Cn display significant depressions in both serum zinc and iron concentrations compared to controls; maximum depressions occurred within 9–12 hr. When 2-hr serum from these intoxicated or infected rats was obtained,

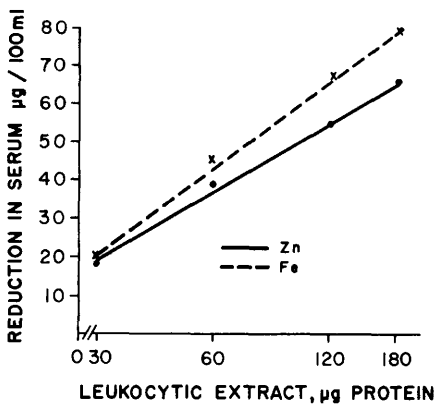

FIG. 1. The effect and relationship between serum zinc and iron reductions and the doses of leukocytic extract administered: Each point represents a minimum of 10 rats. Endotoxin-tolerant control serum zinc = 138 ± 5 µg/100 ml and serum iron = 144 ± 5 µg/100 ml.

filtered, and administered to respective groups of normal rats, significant depressions in serum zinc and iron concentrations were observed in the recipient rats with maximal depressions being accelerated on an average of 2–3 hr in these recipient animals (Table I). However, if the 2-hr serum from the donors was heated at 90° for 30 min prior to being administered, no significant decreases in either serum metal concentration were observed in the recipients.

Figure 1 illustrates the effect of crude leukocytic extract or mediator prepared from the PMN leukocytes of rat peritoneal exudates on serum zinc and iron concentrations of endotoxin-tolerant rats. This experiment not only confirms the fact that the leukocytic extracts mediate an alteration in zinc and iron metabolism, but also that the decreases in their serum concentrations were linear to the logarithm of the doses of extract administered.

A comparison of the effects and activity of equal amounts of crude endogenous mediator, butanol–methanol extracted mediator, and 3 different molecular wt fractions on serum zinc and iron concentrations are shown in Table II. Both the crude and the extracted mediator induced significant decreases in serum zinc and iron concentrations. Since equal doses of protein were injected, the butanol–methanol extracted preparation demonstrated a higher specific activity than the crude mediator, thus indicating partial purification. Of the 3 fractions of different molecular weight ranges tested, the highest specific activity was observed in the 10,000 to 30,000 mol wt range. The less than 10,000 mol wt range also induced decreases in both serum zinc and iron, which were only barely significant. Although not significant, the fraction greater than 30,000 mol wt produced slight decreases in these two serum metals. Heating any of the above preparations at 90° for 30 min prior to injection inactivated their ability to reduce serum zinc or iron concentrations.

Discussion. The data obtained from the transfer experiments in this study using a variety of inflammatory stimuli suggest that the observed decreases in serum zinc and iron

TABLE II. Comparison of the Effects of Crude Endogenous Mediator, Butanol–Methanol Extracted Mediator, and Different Molecular Weight Fractions of the Crude Mediator on Serum Zinc and Iron Concentrations in Endotoxin Tolerant Rats.

Treatment (ip)	Serum zinc (µg/100 ml)[a]	Serum iron (µg/100 ml)[a]
Endotoxin-tolerant control	138 ± 5	144 ± 5
Crude mediator (60 µg of protein)[c]	99 ± 4[b]	97 ± 7[b]
Butanol–Methanol extracted[c] mediator (60 µg of protein)	78 ± 3[b]	67 ± 8[b]
60 µg of protein, > 30,000 mol wt[c]	121 ± 4	129 ± 7
60 µg of protein, 10,000–30,000 mol wt[c]	76 ± 5[b]	66 ± 3[b]
60 µg of protein, < 10,000 mol wt[c]	118 ± 2[b]	114 ± 8[b]

[a] Means of a minimum of 10 animals ± SE.

[b] $p < .01$, compared to controls.

[c] Zinc and iron depressing effect lost when heated 90° for 30 min.

concentrations are mediated as part of the host response via a common heat labile endogenous mediator released, in part, by PMN leukocytes. The rapid appearance of this endogenous mediator in the circulation may be one of the earliest detectable responses of the host to an infection or other inflammatory stress.

Endogenous mediator obtained from the PMN leukocytes of rat peritoneal exudates produced dose-dependent reductions in both serum zinc and iron concentration within the protein range tested. This not only confirms the observations of Kampschmidt *et al.* (10) for plasma iron, but also demonstrates a dose dependency in serum zinc which closely parallels that of iron. Such a dose dependency of serum zinc and iron reductions to an intermediate could be indicative of the severity of an inflammatory response. Pekarek *et al.* (3, 4), while studying volunteers with either bacterial or viral infections, suggested a possible relationship between the severity of illness, the degree of serum zinc, and iron depression.

Although partial purification of the mediator using the butanol–methanol method of Rafter *et al.* (14) for endogenous pyrogen, does increase the specific activity of the mediator, it still remains uncertain if the endogenous mediator of serum zinc and iron depression is the same as endogenous pyrogen. When based on the protein content of the extracted material, one would expect an increase in specific activity as great as that shown for endogenous pyrogen on fever production (14, 16). Thus a certain amount of the metal-depressing mediator may be lost in the purification procedure attempted by us.

However, from the preliminary data presented in Table II, it does appear that the endogenous mediator, based on activity, is in a molecular weight range (10,000–30,000) similar to that of endogenous pyrogen (14, 16). The overlap in activity between the fraction below 1,000 mol wt and that above 30,000 mol wt is most likely due to the retention sensitivity of the filtration membranes employed.

The results of this investigation characterize the endogenous mediator isolated from PMN leukocytes as a heat labile, nondialyzable, low molecular weight protein, which is soluble to some extent in methanol. Although this mediator is similar in many respects to endogenous pyrogen, it remains uncertain whether they are one and the same. This can only be resolved by the further purification of these substances.

Summary. Serum zinc and iron concentrations were shown to be decreased significantly by a common heat labile endogenous mediator present in the serum of animals within 2 hr after the administration of a variety of inflammation inducing agents. The endogenous mediator obtained from PMN leukocytes of peritoneal exudates produced significant dose-dependent reductions in both serum zinc and iron concentrations. Preliminary data characterize the mediator as a heat labile, nondialyzable, low molecular weight protein (10,000–30,000 mol wt).

1. Cartwright, G. E., Lauritsen, M. A., Jones, P. J., Merrill, I. M., and Wintrobe, M. M., J. Clin. Invest. **25**, 65 (1946).

2. Vikbladh, I., Scand. J. Clin. Lab. Invest., Suppl. (n2) 3, 1 (1951).

3. Pekarek, R. S., Bostian, K. A. L., Bartelloni, P. J., Calia, F. M., and Beisel, W. R., Amer. J. Med. Sci. **258**, 14 (1969).

4. Pekarek, R. S., Burghen, G. A., Bartelloni, P. J., Calia, F. M., Bostian, K. A., and Beisel, W. R., J. Lab. Clin. Med. **76**, 293 (1970).

5. Halsted, J. A., and Smith, J. C., Jr., Lancet **1**, 322 (1970).

6. Kampschmidt, R. F., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **110**, 191 (1962).

7. Pekarek, R. S., and Beisel, W. R., Appl. Microbiol. **18**, 482 (1969).

8. Pekarek, R. S., Proc. Soc. Exp. Biol. Med. **136**, 584 (1971).

9. Beisel, W. R., and Pekarek, R. S., Int. Rev. Neurobiol. in press.

10. Kampschmidt, R. F., Upchurch, H. F., and Eddington, C. L., Proc. Soc. Exp. Biol. Med. **132**, 817 (1969).

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

12. Kampschmidt, R. F., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **133**, 128 (1970).

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

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

15. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

16. Kozak, M. S., Hahn, H. H., Lennarz, J. L., and Wood, W. B., Jr., J. Exp. Med. **127**, 341 (1968).

Received June 17, 1971. P.S.E.B.M., 1971, Vol. 138.