Hypoferremia Produced by Plasma from Endotoxin-Treated Rats (34314)

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After an injection of endotoxin, the plasma iron concentration of the rat reaches a minimum between 8 and 12 hr and then slowly returns to normal (1). With repeated daily injections of endotoxin, a tolerance is developed (2). This leaves unexplained the chronic depression of plasma iron concentration that frequently occurs in animals with infection or during tumor growth (3).

Animals injected daily with bacterial endotoxin develop a tolerance to its pyrogenic action (4). There is now considerable evidence that endotoxin produces fever indirectly by liberating an endogenous pyrogen from the tissues of the host which will cause fever in an endotoxin-tolerant animal (5). One of the major sources of this pyrogen seems to be the leukocytes; and when endotoxin was incubated with granulocytes from peritoneal exudates, an endogenous pyrogen was released *in vitro* (6). Furthermore, endotoxins failed to cause fever in rabbits made granulocytopenic with nitrogen mustard (7). A similar mechanism may be responsible for the lowering of plasma iron concentration in the rat, since it was recently shown that the decrease in the concentration of plasma iron after the injection of endotoxin was greater in normal rats than in those rendered leukopenic by the administration of nitrogen mustard (8). Leukocytic pyrogen prepared from polymorphonuclear leukocytes also depressed plasma iron in endotoxin-tolerant rats (8).

Endogenous pyrogen has been demonstrated in the blood stream approximately 2 hr after injection of endotoxin in rabbits and dogs (9–11). This pyrogen was a protein which would produce a rapid increase in body temperature in endotoxin-tolerant animals. Some suggestion of a similar factor that will produce hypoferremia has been found in rats injected with endotoxin (2). The activity in the plasma 2 hr after injection of endotoxin could be destroyed by heating at 90° for 30 min (2). Numerous investigations on fever have indicated that endotoxin mixed with serum has many characteristics which resemble endogenous pyrogen (12–16). The purpose of the present investigation was to partially purify the hypoferremia-producing factor in the plasma of rats 2 hr after endotoxin and compare it with endotoxin in serum and leukocytic pyrogen.

Materials and Methods. All measurements of plasma iron concentration were in female Holtzman rats weighing 180–200 g. Blood was collected from the heart of anesthetized rats with a heparinized syringe and the plasma separated by centrifugation. The plasma iron concentration was determined by the 2,-2′,2″-terpyridine method as described by Schade *et al.* (17).

The endotoxin was *Escherichia coli* 055: B5, lot 460830, from Difco Laboratories, Detroit, Michigan. Tolerance to endotoxin was produced by giving 1 μg ip for 10 daily injections.

At varying intervals after intravenous injection of 50 μg of endotoxin, the hypoferremia-producing factor in plasma was partially purified by treatment with phenol. Varying concentrations of phenol were achieved by adding the appropriate amount of a 100 g/100 ml of phenol solution to the plasma sample. After the aqueous layer stood for 10 min at room temperature, it was separated by centrifugation and dialyzed against pyrogen-free distilled water at 4° until free of phenol. To check the biological activity, an aliquot equivalent to 0.5 ml of the original plasma was injected intraperitoneally into normal rats. The amount of protein in the aqueous layer, after treatment with phenol, was mea-

TABLE I. Effect of Adding Varying Phenol Concentrations to Plasma Obtained 2 hr after Injecting 50 μg of Endotoxin on the Protein Remaining in the Aqueous Phase and Its Activity in Lowering Plasma Iron Concentration.[a]

Phenol (%)	Protein (mg/ml)	Plasma iron[b] (μg/100 ml)
0	82.2 ± 4.3[c]	51 ± 6[c]
10	4.9 ± 1.3	66 ± 6
12	3.2 ± 0.9	75 ± 25
14	2.6 ± 0.7	45 ± 7
16	1.1 ± 0.6	90 ± 11
18	0.6 ± 0.2	143 ± 18
20	0.2 ± 0.2	146 ± 16
30	—	198 ± 15
40	—	208 ± 19
60	—	225 ± 21
Control	—	256 ± 12

[a] Plasma used, with the exception of the control, was obtained from rats 2 hr after they received an iv injection of 50 μg of *E. coli* endotoxin.

[b] Normal rats received an ip injection of 0.5 ml of treated plasma. Plasma iron was determined 16 hr later.

[c] Mean ± SE for 4 determinations.

sured with Folin phenol reagent by the method of Lowry *et al.* (18).

Antibody to endotoxin was produced in rabbits. Each rabbit received twice weekly iv injections of endotoxin on the following schedule of increasing dosage: 125, 250, 250, 500, 750, and 1000 μg. One week after the last injection the serum antibody titer was checked by the bentonite flocculation procedure described by Wolff *et al.* (19), in which 600 μg of endotoxin had been adsorbed per 10 ml of the stock bentonite preparation. The rabbits were then bled by heart puncture, the serum was separated and stored frozen in small portions. One vol. of the antiserum was mixed with 10 vol of rat plasma, obtained 0 or 2 hr after endotoxin, and incubated at 37° for 15 min prior to ip injection to test the biological activity.

Results and Discussion. In previous studies whole plasma from rats, which had received injections of endotoxin, was used to produce hypoferremia (2). This made it difficult to determine whether the results obtained were due to an endogenous factor or to protein interactions with endotoxin. Since endotoxin which is not associated with protein remains in the aqueous phase upon extraction with phenol (20), the effects of extracting postendotoxin plasma with varying concentrations was investigated. The results, given in Table I, showed most plasma proteins to be removed with low phenol concentrations, whereas the hypoferremia-producing activity remained in the aqueous phase up to a phenol concentration of 14%.

In an attempt to determine if this activity was due to an endogenous protein or an association between endotoxin and protein, the plasma was treated with 14% phenol at varying times after endotoxin (Table II). After phenol treatment, half of each plasma sample was heated at 90° for 30 min. The aqueous phase of phenol-treated plasma retained most of its hypoferremia-producing activity during the first 4 hr after injecting 50 μg of endotoxin. None of the hypoferremia-producing activity of the plasma obtained immediately after enotoxin injection was inactivated by heating. In a very few min-

TABLE II. Effect of Heat upon Hypoferremia-Producing Activity of Phenol-Treated (14%) Plasma Obtained at Various Times after an iv Injection of 50 μg of Endotoxin.

Plasma used for treatment (min after endotoxin)	No. of trials	Plasma iron (μg/100 ml)[a]	
		No heat	Heated[b]
Control	10	247 ± 13	263 ± 11
0[c]	17	56 ± 5	56 ± 5
7.5	8	67 ± 5	89 ± 8
15	15	70 ± 7	172 ± 13
30	21	28 ± 9	178 ± 19
60	17	88 ± 8	201 ± 21
120	15	84 ± 7	229 ± 18
180	6	95 ± 10	225 ± 23
240	4	93 ± 12	253 ± 29
360	6	183 ± 14	—
Control + 3 μg of endotoxin/ml[d]	8	112 ± 11	104 ± 18

[a] Measured 16 hr after the rats received an ip injection of 0.5 ml of phenol-treated plasma.

[b] Heated at 90° C for 30 min.

[c] Five to 30 sec after injection.

[d] Control plasma incubated at 37° for 2 hr with 3 μg/ml of *E. coli* endotoxin.

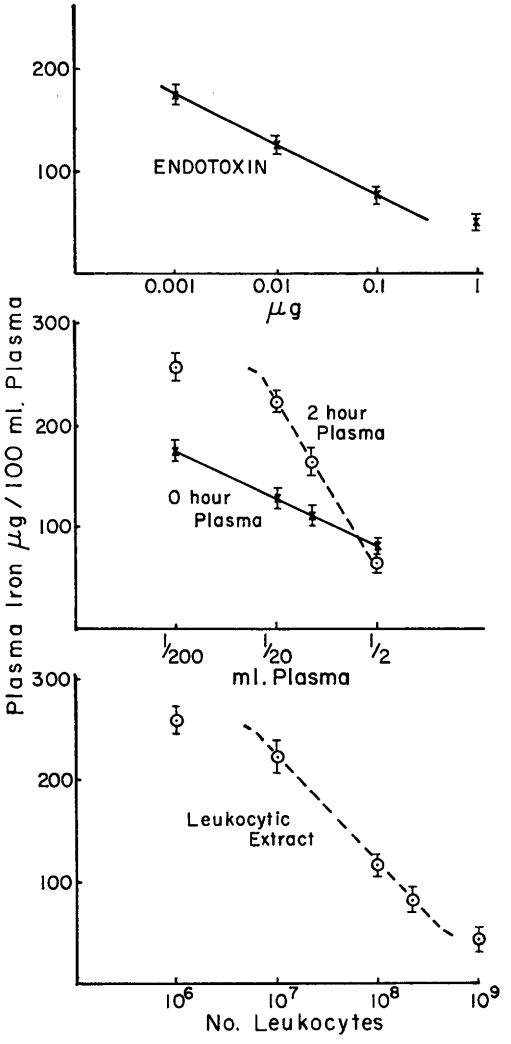

FIG. 1. Log dose curve for hypoferremia produced in rats by injection of plasma from rats either immediately or 2 hr after an iv injection of 50 μg of endotoxin; these are compared to the curves obtained when either endotoxin (top) or leukocytic extract from rat peritoneal leukocytes (bottom) were injected.

utes, however, a portion of the activity was destroyed when heated at 90° for 30 min. Between 2 and 4 hr after endotoxin almost all of the activity in the plasma was lost after heating. This inactivation with heat did not occur when endotoxin was incubated *in vitro* for 2 hr at 37° with rat plasma.

A further indication that the activity found in the plasma at 2 hr after endotoxin was different than endotoxin mixed with plasma is shown in Fig. 1. The plots of the log dose curve for the 0-hr plasma and endotoxin had identical slopes. By contrast the curve for the 2-hr plasma had a steep slope similar to that obtained upon using an extract from polymorphonuclear leukocytes (8).

When the 2-hr plasma was injected into rats which were tolerant to 1 μg of endotoxin, it produced no lowering of plasma iron. The pretreatment and the challenge dose were both given ip (21). The 2-hr plasma also produced a tolerance when repeated daily injections were given. The activity of the 2-hr plasma was not destroyed by incubation with proteolytic enzymes. It was, therefore, unlike the leukocytic extract that was effective in rats, tolerant to endotoxin and could be destroyed by proteolytic enzymes (8).

Since the ineffectiveness of 2-hr plasma in endotoxin-tolerant rats indicated that endotoxin was still present, confirmation was sought through the use of specific antiserum. When *E. coli* endotoxin (1 μg) was incubated with this antiserum and injected into normal rats, no decrease in plasma iron was observed. This *E. coli* antiserum seemed to be specific since it would not inactivate endotoxin prepared from *Salmonella typhimurium*. The effects of this antiserum on the plasma obtained either immediately after endotoxin injection or 2 hr later are shown in Table III. These results also indicate that the plasma 2 hr after injection still contained endotoxin.

One possible explanation for the results would be that in a very few minutes after injection of endotoxin in the rat a protein is formed which complexes with the endotoxin. Apparently this was a protein not normally present in large quantities, since a similar complex was not formed when endotoxin and rat plasma were incubated together *in vitro*. With low concentrations of phenol, this complex remains in the aqueous phase, but at phenol concentrations above 30% most of it was precipitated. Heating this protein–endotoxin complex apparently altered the protein so that it was removed rapidly by the reticuloendothelial system. The formation of

TABLE III. Effect of Endotoxin Antibody on the Hypoferremia-Producing Activity of Rat Plasma Obtained 0 and 2 hr after Endotoxin Injection.

No. of trials	Rat plasma time after endotoxin (hr)	Rabbit serum used[a] (0.1 ml/ml of plasma)	Plasma iron[b] (μg/100 ml)
6	0	None	105 ± 8[c]
9	2	None	79 ± 10
6	0	Normal	107 ± 10
6	2	Normal	168 ± 10
6	0	Antiendotoxin	200 ± 5
6	2	Antiendotoxin	235 ± 31

[a] Rabbit serum, 0.1 vol, was incubated at 37° for 15 min with plasma samples obtained at 0 and 2 hr after injection of 50 μg/rat.

[b] Plasma iron was measured 16 hr after injection of 0.25 ml of the mixtures of 0 rat plasma and rabbit serum or 0.5 ml of the 2-hr rat plasma and rabbit serum. (Less 0 plasma was used so that it would be about as active as the 2-hr plasma.)

[c] Mean ± SE.

a protein–endotoxin complex, which remains in the plasma for 5–6 hr after an endotoxin injection will increase the difficulty of demonstrating the formation of an endogenous factor in rat plasma after endotoxin injection. Endogenous factors from leukocytes can also produce hypoferremia (8), but as yet there is no conclusive evidence that these endogenous factors are intermediates in the production of hypoferremia in the rat by endotoxin.

Summary. After intravenous injection of endotoxin in the rat, a hypoferremia-producing factor remained in the plasma for 5–6 hr. This factor was different from endotoxin incubated *in vitro* with rat plasma. Endotoxin, however, was still present, suggesting the *in vivo* formation of a protein–endotoxin complex. The possibility of an intermediate being involved in the production of hypoferremia in the rat after endotoxin injection was discussed.

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