Biologic Relationship of Endotoxin and Other Toxic Proteins.* III. Effect of Ferrous Iron on Toxicity of Endotoxin and Snake Venom. (28987)

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The study of the nonspecific resistance to toxins has been approached in a variety of ways and has generated a wide range of hypotheses regarding the systems involved and their action. The reticuloendothelial system (RES) has been considered by many(1) to play a major role in nonspecific resistance phenomena, and it is prominent in the theory of Heilmeyer *et al*(2) regarding the fundamental importance of serum iron in resistance to toxins. Following the injection of a variety of toxins, serum iron levels are decreased(3), the deposition of iron as hemosiderin in the RES is increased(2), and absorption of iron from the intestine is increased(4-6). Recently, Kampschmidt and Upchurch(7) showed that a single intravenous injection of microgram quantities of endotoxin lowers plasma iron levels in rats.

Heilmeyer and associates(2,8) demonstrated that incubation of a variety of endotoxins and exotoxins with ferrous iron protected animals subsequently injected with the toxins from their lethal effects. Janoff and Zweifach(9) extended these studies to the endotoxin of *Escherichia coli,* and demonstrated protection (using the same incubation procedures) against the lethal effects of endotoxin, as well as the local and generalized Schwartzman reactions, but not against the characteristic pyrogenic response.

There have been fewer *in vivo* experiments relating iron to nonspecific resistance against toxins. Hettche(10) showed increased resistance in animals pretreated with multiple doses of iron; and Heilmeyer *et al*(2) found that Pyrexal-treated guinea pigs which were given additional parenteral iron were resistant to lethal doses of diphtheria toxin over extended periods.

In our earlier experiment(11), studies of the detoxifying effect of *in vitro* incubation of toxins with ferrous iron were extended to the venom of *Agkistrodon piscivorus* (water moccasin snake). We demonstrated that incubation of an LD_{100} of this venom with 5 or 10 mg of ferrous sulfate prior to its injection in rabbits offered significant protection against the lethal effects of the venom.

E. coli endotoxin has been found to produce hypersusceptibility to sublethal doses of some snake venoms, resulting in immediate death of the animals upon challenge with the venom. Further experiments(12) defined the period of hypersusceptibility to venom *(Agkistrodon piscivorus)* as 3-4 days following intravenous administration of endotoxin, with a brief latent period following the injection. This sequence, and the known facts regarding alterations in serum iron following toxin administration, suggested that a period of altered iron metabolism might be involved in hypersusceptibility to venom induced by endotoxin treatment.

The present experiments were carried out to determine whether incubation of ferrous iron with endotoxin prior to its injection would inhibit the susceptibility to venom; and if similar incubation of iron with snake venom would block the lethal action of sublethal doses of venom in endotoxin-treated animals. Parallel *in vivo* studies were also done, involving a single injection of iron sulfate at varying intervals between the endotoxin and venom injections. Ferrous sulfate was also given intravenously prior to injection of lethal doses of venom in normal animals, an *in vivo* study paralleling the earlier *in vitro* experiment.

An incidental finding of increased susceptibility to toxic effects of ferrous sulfate in endotoxin-treated animals was also investigated, using varied doses of endotoxin and ferrous sulfate.

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TABLE I. Effect of Incubation of Ferrous Sulfate and Endotoxin on EIHS.

Experimental plan*	Quantity of ferrous sulfate	Deaths† 1 hr	Deaths† 24 hr
100 γ of endotoxin, previously incubated with varying amounts of $FeSO_4$, followed in 2 hr by 500 γ of snake venom	125 γ	9/10	9/10
	5 mg	4/5	4/5
100 γ of endotoxin, incubated without ferrous sulfate, followed in 2 hr by 500 γ of snake venom	—	11/15	11/15
100 γ of endotoxin, incubated without ferrous sulfate	—	0/10	1/10
500 γ of snake venom	—	1/10	1/10

* All toxins administered intravenously.
† No. of deaths within period specified (following snake venom challenge) over total number of animals.

Materials and methods. Escherichia coli endotoxin (Difco Laboratories, Detroit, lot no. 0127.B8) and dehydrated water moccasin *(Agkistrodon piscivorus)* venom, obtained from the Ross Allen Reptile Institute, Silver Springs, Fla., were used in these experiments. The vehicles in all experiments were pyrogen-free saline solutions from Cutter Laboratories, Berkeley. Reagent grade ferrous sulfate was used as the source of ferrous iron ($FeSO_4 \cdot 7H_2O$, molecular weight = 278.02).

The rabbits were albino hybrids, weighing 1 kg, obtained from a single local breeder, fed Purina rabbit pellets, and given water *ad libitum.*

EIHS models. The endotoxin-induced hypersusceptibility state (EIHS) was produced by the intravenous injection of 100 γ of endotoxin, with challenge by snake venom (500 γ intravenously) after 1 or 2 hours. This resulted in immediate death of a majority of the rabbits. Time of death was recorded in all experiments. The procedures are referred to as the 1-hour EIHS model and the 2-hour EIHS model, depending on the interval between injections.

Incubation procedure. Incubation of the toxins with iron sulfate was carried out just before injection. The samples were kept at 37°C for 6 hours in a warm air incubator. All samples were completely reconstituted by gentle stirring prior to injection.

Results. Effect of incubation of ferrous sulfate and endotoxin on EIHS. Ferrous iron incubated with gram-negative bacterial products prior to injection has been shown to protect rabbits against many of the toxic properties of endotoxin(9). In this group of experiments, varying quantities of ferrous sulfate were incubated with 100 γ of endotoxin, as outlined earlier.

Two dose levels of ferrous sulfate, 200 γ and 1 mg, failed to inhibit the endotoxin-induced susceptibility to the lethal effects of 500 γ of snake venom given 1 hour later. Four of 10 (200 γ) and 6 of 10 (1 mg) rabbits died, compared with 6 of 10 of the control group.

Table I shows the results, using the 2-hour EIHS model and incubation of endotoxin with 125 γ and 5 mg of ferrous sulfate. The venom administration was consistently lethal within an hour in both the experimental and control groups.

Ferrous iron does not protect against EIHS when it is incubated with the endotoxin prior to injection and the venom challenge is offered 1 or 2 hours later.

Effect of incubation of ferrous sulfate and snake venom on demonstration of EIHS. Previous experiments showed that incubation of 5 mg of ferrous iron with an LD_{100} of water moccasin venom prior to its injection in a normal rabbit protected about half of the animals against the lethal effects of the venom(11). Whether the factor or factors in this venom which elicit the EIHS can be neutralized with ferrous sulfate was studied in this group of experiments.

Using the 1-hour model, prior incubation of the venom with 500 γ, 1 mg, and 5 mg of iron sulfate protected against the lethal effect of the venom in a significant number of animals. As shown in Table II, there were no losses with the 5 mg dose, compared to 8 of 10 in the control group which received 500 γ

TABLE II. Effect of Incubation of Ferrous Sulfate and Snake Venom on Demonstration of EIHS.

Experimental plan*	Quantity of ferrous sulfate	Deaths† 1 hr	Deaths† 24 hr
100 γ of endotoxin, followed in 1 hr by 500 γ of snake venom, previously incubated with varying quantities of ferrous sulfate	500 γ	2/10	2/10
	1 mg	3/10	4/10
	5 "	0/10	0/10
100 γ of endotoxin, followed in 1 hr by 500 γ of snake venom, previously incubated without ferrous sulfate	—	8/10	8/10
500 γ of snake venom, incubated without ferrous sulfate	—	0/5	0/5
100 γ of endotoxin	—	0/10	1/10

* All toxins administered intravenously.
† No. of deaths within period specified (after administration of snake venom) over total number of animals.

TABLE III. Effects of Administration of Iron Sulfate on Susceptibility to Snake Venom.

Intravenous iron sulfate (mg)	Interval between injections (min.)	Intravenous snake venom (mg)	Deaths* 1 hr	Deaths* 24 hr
10	30	8	3/8	5/8
25	1	8	2/6	4/6
—	—	8	14/14	
10	—	—	0/10	0/10
25	—	—	0/10	0/10
100	—	—	8/10	8/10

* No. of deaths within period specified (following the only injection in control animals, and following second injection in experimental groups) over total number of animals.

of venom without iron. Two of 10 and 3 of 10 died in the 500 γ and 1 mg dose groups respectively.

Thus, prior incubation of venom with ferrous sulfate offers significant protection against the lethal effects of venom in the endotoxin-treated animals.

Effects of in vivo *administration of ferrous sulfate on susceptibility to venom.* In Table III can be seen the results of experiments using intravenous ferrous sulfate to protect against an LD_{100} of water moccasin venom, 8 mg. Twenty-five mg of ferrous sulfate injected 1 minute before the venom protected 4 of 6 rabbits from immediate death. Two additional animals died within 24 hours, and 2 others survived.

Protection was also noted in a group of animals given 10 mg of ferrous sulfate 30 minutes preceding the LD_{100} of venom. Three died within an hour, 2 additional animals 1-24 hours after administration of venom, and 3 survived the 24-hour observation period.

One of the control groups in this experiment received 100 mg of ferrous sulfate alone which proved to be a toxic dose. Eight of 10 rabbits died immediately. The 10 and 25 mg doses were not toxic in normal animals.

Iron sulfate, given intravenously prior to administration of lethal doses of snake venom, protects some animals against the lethal effects of venom for a limited period and others indefinitely.

Effects on EIHS of administration of ferrous sulfate between injections of toxins. Following the demonstration of protection against the lethal effects of snake venom in both normal and hypersusceptible animals when ferrous sulfate and venom were incubated prior to administration, experiments were undertaken to determine whether direct administration of ferrous sulfate between the injections of endotoxin and venom would protect.

As shown in Table IV, with the 1-hour EIHS model, a protective effect was suggested when 5 mg of iron sulfate was given 1 minute after endotoxin. Five of 10 animals died immediately, compared with 8 of

TABLE IV. Effects of Administration of Iron Sulfate on Susceptibility of Endotoxin-Treated Rabbits to Snake Venom.

Intravenous endotoxin (γ)	Endotoxin-$FeSO_4$ interval (min.)	Intravenous $FeSO_4$ (mg)	Endotoxin-venom interval (hr)	Intravenous venom (γ)	Deaths* 1 hr	Deaths* 24 hr
100	1	5	1	500	5/10	5/10
100	10	10	1	500	4/10	6/10
100	50	10	1	500	4/10	4/10
100	10	10	2	500	12/20	12/20
100	—	—	1	500	8/10	8/10
100	—	—	2	500	20/20	20/20

* No. of deaths within period specified (following snake venom injection) over total number of animals.

10 of the controls. Similarly, groups of animals given 10 mg of ferrous sulfate, 10 and 50 minutes after endotoxin, demonstrated low-grade protection. In both groups, 4 of 10 animals died.

With the 2-hour EIHS model and a larger group of rabbits, the protective effects were seen more clearly. Ten mg of ferrous sulfate was injected 10 minutes after endotoxin administration. Twelve of 20 iron-treated animals died; all 20 of the control EIHS group died.

It can be seen from the Table that significant protection against the lethal effects of snake venom in hypersusceptible rabbits is afforded by injection of ferrous sulfate after the endotoxin. Both 5 and 10 mg doses were effective, and the interval between endotoxin and iron administration (ranging from 1-50 minutes) did not appear to be significant for immediate susceptibility.

Effect of endotoxin-pretreatment on susceptibility of rabbits to iron sulfate. In some of the experiments involving direct intravenous administration of ferrous sulfate, it was suggested that endotoxin-treated animals are more susceptible to toxic effects of iron. Further studies were done to determine whether this is a consistent finding.

Varied doses of iron sulfate were administered to rabbits pretreated 15-30 minutes earlier with either 100 or 500 γ of endotoxin. The results, summarized in Table V, confirm the earlier suggestion of increased susceptibility. Ferrous sulfate in untreated animals was nontoxic until levels of 100 mg were injected. Toxicity was reflected in marked hyperactivity and convulsions, and early death.

Hypersusceptibility was evident in the endotoxin-treated animals. In the group which received 100 γ of endotoxin, 25 mg of ferrous sulfate was lethal to 4 of 6 rabbits within 24 hours, compared to one death among 10 controls which received only endotoxin. No animals died when 25 mg of ferrous sulfate was given intravenously alone.

In animals treated with 500 γ of endotoxin, 3 of 10 control rabbits died within 24 hours. A dose of 5 mg of ferrous sulfate resulted in deaths of 2 of 7 rabbits, and 25 mg of ferrous sulfate killed 8 of 10 rabbits.

The deaths in the hypersusceptible group were late deaths, unlike those of the normal animals receiving lethal doses of iron sulfate, and similar to those of normal animals receiving lethal doses of endotoxin alone. Although the increased susceptibility to iron in endotoxin treated animals was minimal in comparison to the venom susceptibility, it appears to be significant.

Discussion. Definite protection with fer-

TABLE V. Increased Susceptibility to Iron Sulfate in Endotoxin-Treated Rabbits.

Intravenous endotoxin (γ)	Intravenous iron sulfate* (mg)	Deaths† 1 hr	Deaths† 24 hr
100	25	0/6	4/6
500	5	0/7	2/7
500	25	1/10	8/10
100	—	0/10	1/10
500	—	0/10	3/10
—	5	0/10	0/10
—	10	0/10	0/10
—	25	0/10	0/10
—	100	8/10	8/10

* Administered 15-30 min. following endotoxin.
† No. of deaths within period specified (after iron sulfate injection) over number of animals.

rous sulfate against the lethal effects of snake venom has been shown in these experiments, both in normal and endotoxin-pretreated animals. Protection was evident when iron was administered intravenously between the endotoxin and snake venom injections, and also when venom had been incubated with ferrous sulfate prior to injection.

Incubation of endotoxin with ferrous iron proved to have no protective effect against endotoxin-induced hypersusceptibility to snake venom. Since incubation with iron has pronounced effects on the lethal and tissue-necrotizing capacities of endotoxin(9), this is another instance of dissociation of the effects of endotoxin. Earlier studies(9) had also shown that the incubation with iron does not alter the pyrogenic effects of endotoxin.

Pyrogenicity and EIHS to venom also vary independently in some circumstances. In a series of experiments on the effect of route of injection of endotoxin on hypersusceptibility to venom(12), both intradermal and intraperitoneal endotoxin produced hypersusceptibility. Intradermal endotoxin is not pyrogenic(13,14). Intraperitoneal endotoxin produces the characteristic temperature curve during the first few hours of injection, when there is almost no increase in the normal susceptibility to venom (the peak of hypersusceptibility *via* this route is reached at 48 hours).

Further experimentation is needed with incubation of endotoxin with higher doses of iron before it is established that the iron does not protect against the pyrogenic response to endotoxin. At present, however, the data appear to confirm the dissociation of pyrogenicity from the EIHS and Shwartzman activity of endotoxin. This apparent separation in principles may reflect two or more series of reactions, proceeding at different rates, but initiated by a common factor. Conversely, this may represent the biologic expression of two or more separate components of the endotoxin complex.

The mechanism involved in the detoxifying activity of iron is in question. Heilmeyer *et al*(2) proposed an interaction of iron and the reticuloendothelial system as a basis for nonspecific resistance of animals. They suggested that hemosiderin in the cells of the RES absorb toxins from the circulation and detoxify them until specific mechanisms, *i.e.*, formation of antitoxins, come into play. Janoff and Zweifach(9), on the other hand, have suggested that iron acts as a colloidal agent in increasing the activity of the RES, already known to play a role in nonspecific resistance. Using a saccharated colloidal iron (Proferrin) as pretreatment, they have reported protection against both local and generalized Shwartzman reactions in one group of animals. They found Proferrin to be superior to Thorotrast as a stimulant of the RES, and suggest that this is probably true of other forms of iron. The protection against toxins afforded by iron may not be related to RES activity. The protective action could represent combination of the iron with specific components of the toxin complex.

It is clear from the venom experiments that the detoxifying action of previously injected ferrous iron on the venom introduced is immediate, since the characteristic venom death occurs within a few minutes in both normal and endotoxin-treated animals. Since injected ferrous sulfate had a significant detoxifying effect on an LD_{100} of venom given one minute later, it is also evident that there is no significant lag in the establishment of detoxifying capacity of iron in the bloodstream. This raises a question as to the importance of the 6-hour incubation period used in prior *in vitro* studies and carried over into this series of experiments.

The finding of hypersusceptibility to subtoxic doses of iron sulfate in endotoxin-treated animals is an enigma, and it is not known whether it is related to endotoxin-induced hypersusceptibility to some bacteria, viruses, and snake venoms. Iron metabolism is complex, and requires oxidative metabolism of the liver cell for release to the plasma of the iron binding protein, transferrin. Specifically, the finding may be related to the need for continuous synthesis of adenosinetriphosphate for the incorporation and release of iron from ferritin(15). The late deaths observed in these experiments were characteristic of endotoxin, suggesting that

iron interfered ultimately with the successful detoxification of the endotoxin.

It has been questioned in earlier work on endotoxin-induced susceptibility to venom whether the toxic principle of the venom operating in susceptible animals is the same as that operating in the normal animal. The fact that ferrous sulfate protects against venom challenge in both groups suggests this possibility, although iron has been shown to offer nonspecific protection to a range of toxins. Further experiments are being conducted with venom fractions in an effort to separate the lethal factors in the two situations if this is possible.

Summary. 1. Incubation of ferrous sulfate with endotoxin for 6 hours at 37°C prior to injection did not alter the endotoxin-induced-hypersusceptibility-state (EIHS) to water moccasin venom in rabbits. 2. Incubation of ferrous sulfate with snake venom under the same conditions prior to injection gave significant protection against the lethal effects of the venom in endotoxin-pretreated rabbits. 3. Intravenous ferrous sulfate protected a significant number of animals against the lethal effects of snake venom when administered between the preparing endotoxin and the challenging dose of moccasin venom. 4. Prior intravenous ferrous sulfate also resulted in protection against an LD_{100} of moccasin venom in normal rabbits. 5. Finally, it was shown that administration of large, but nontoxic, doses of ferrous sulfate to endotoxin-pretreated animals resulted in the delayed death of most of the animals.

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Comparison of Sympathetic Blocking Drugs in Prevention of Lethal Effects of Endotoxin.* (28988)

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Shock which occurs during bacteremia, particularly bacteremia caused by gram-negative enteric organisms, is an increasingly serious clinical problem. The results of therapy have been disappointing. In a clinical study based on 169 patients with bacterial shock recently reported by our group, mortality exceeded 80%(1). The development and progression of this type of shock is related to the liberation of endotoxin from the bacterial cells(2,3). Sympatholytic drugs, especially

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