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BRIEF COMMUNICATION

Behavioral Conditioning of Endotoxin-Induced Plasma Iron Alterations

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EXTON, M. S., D. F. BULL, M. G. KING AND A. J. HUSBAND. *Behavioral conditioning of endotoxin-induced plasma iron alterations.* PHARMACOL BIOCHEM BEHAV **50**(4) 675-679, 1995.—The cascade of physiologic mechanisms in response to infection, the acute-phase response, is recognized as playing a major role in host defence. One such response is the hypoferremia that is consistently reported to occur during bacterial infection. This study aimed to determine whether the alterations in plasma iron were conditionable using the conditioned taste aversion (CTA) paradigm. The regime involved the pairing of a novel-tasting saccharin solution with bacterial endotoxin. Seven days after the initial pairing of these stimuli (the test day), the saccharin solution was represented. Animals exposed to this condition displayed a significant reduction in the level of plasma iron. Animals treated with an intraperitoneal dose of 400 μg/Kg lipopolysaccharide (LPS) displayed lower conditioned iron levels than rats infused with 100 μg/Kg LPS; however, this difference was not significant. These results showed that in addition to other acute-phase responses (fever and anorexia), plasma iron alterations are able to be manipulated through behavioral manipulations.

Conditioned taste aversion Host defense Acute-phase response Plasma iron Lipopolysaccharide

THE ACUTE-PHASE response is a primary host defence mechanism, and is a reply to host insults such as microbial infection and tissue injury. The response includes a range of events such as fever, sleep alterations, endocrine reactions, altered immune cell functioning, "sickness behavior," and changes in the plasma concentrations of certain trace metals (25). Each of these mechanisms is not an isolated event, but a cascade of reactions designed for optimal immunocompetence and survival capability of the organism.

Research has demonstrated that certain acute-phase parameters, such as fever (2,3,10) and anorexia (9) may be reenlisted using a conditioned taste aversion (CTA) paradigm. The present report examines the conditionability of immune alterations in plasma iron.

Bacterial infection has been demonstrated to alter consistently plasma levels of various trace metals. Administration of endotoxin produces a dramatic decrease in plasma iron in most vertebrates, including rats (15,16,18), mice (35), cattle (6,40), pigs (12), goats (39), avian species (4,37), reptiles (11), rabbits (20,28), guinea pigs (1), and humans (8).

The alteration of plasma trace metals—in particular, the fall in serum iron—is postulated to be an integral mechanism in the host response to infection. Evidence for this position has accumulated via a number of research areas. First, iron is an essential element required for many of the cellular mechanisms involved in the growth of many pathogens. Indeed, many strains of bacteria display retarded growth and replication in an iron-deficient medium (13,41). In contrast, iron-rich plasma potentiates both the growth (7,14,23,29,36) and virulence and lethality (24,33) of numerous bacterial strains.

Furthermore, the decrease in plasma iron levels has been posited to act synergistically with fever development, the production of which is an integral mechanism in the host response to bacteria [see Kluger (22) for a review]. The growth rates of bacteria support this position. When bacteria is grown in an iron-deficient environment, the growth rate of the bacteria is

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considerably lower at the febrile temperature (11,20,21). In addition, organisms administered iron supplements after bacterial infection display higher mortality rates than those allowed to develop hypoferremia (11).

Like the generation of fever, endotoxin-induced hypoferremia is most likely mediated by interleukin-1 (IL-1), which has also been demonstrated to induce hypoferremia (17,19, 42). Moreover, the production of fever is generated within the preoptic anterior hypothalamus (PO/AH) (22). Similarly, hypoferremia in response to endotoxin is mediated at least in part by a central mechanism akin to that which mediates the febrile response (30-32).

Thus, because of the synergistic nature of temperature and plasma trace metal alterations in combating pathogens, the similar mode of action of these mechanisms, and the known conditionability of fever, this study examined the conditionability of plasma iron concentrations. This research demonstrates that we can produce a conditioned decrease in plasma iron in response to bacterial endotoxin.

METHOD

Subjects

We used 46 male rats, ages 100-110 days, at the beginning of the experiment. All rats were of the Australian Albino Wistar inbred strain, and were obtained from the University of Newcastle Psychology Department animal house, from AAEC stock. Animals with a mean weight of 360 ± 15 g were used, and were individually housed in standard wire laboratory cages (18 × 23 × 15 cm high). Cages were kept in an air-conditioned holding room, with an ambient temperature of 22.0 ± 1.0°C. The rats had access to standard laboratory food pellets and tapwater ad lib, except during the water deprivation phase of the experiment. A 12 L : 12 D cycle with lights on at 06:00 h was maintained throughout the experiment.

Apparatus

The conditioned stimulus (CS) used was a 1% saccharin solution. Lipopolysaccharide (LPS) derived from *Escherichia coli* endotoxin (026 : B6; Difco, Sydney, Australia), at a dosage of either 100 or 400 μg/Kg was employed as the unconditioned stimulus (UCS). Pyrogen-free 0.9% saline solution (Newcastle Veterinary Supplies, Newcastle, Australia) was used for the control injections.

The analysis of plasma iron levels was conducted using a Roche Ultimate 7 Iron Kit (Roche Diagnostics, Sydney, Australia), and were run on a Cobas Bio Analyser (Roche Diagnostics). The Roche Iron Kit contained a buffer solution and a chromogen solution. The buffer was composed of sodium acetate (pH 4.5) (200 mmol/l), thiourea (120 mmol/l), hydroxylamine (300 mmol/l), and guanidine hydrochloride (4.5 mol/l). The chromogen contained sodium acetate (200 mmol/l) and ferrozine (39 mmol/l).

TABLE 1

TREATMENT GROUPS

Group	No. of Rats	Conditioning Day CS	Conditioning Day UCS	Test Day CS
SAC/LPS100	8	Saccharin	LPS (100 υg)	Saccharin*
SAC/LPS400	6	Saccharin	LPS (400 υg)	Saccharin*
SAC/LPS100	8	Saccharin	LPS (100 υg)	Water*
WAT/LPS	8	Water	LPS (100υg)	Water*
SAC/SAL	8	Saccharin	Saline	Saccharin*
WAT/SAL	8	Water	Saline	Water*

*Saline control injection, all animals.

Experimental Procedure

For 4 days before experimentation, animals were handled gently to minimize stress throughout the entire experimental paradigm. Animals were then assigned to one of six groups (Table 1). Two groups were administered the experimental condition of a saccharin/LPS pairing. One of these groups received an intraperitoneal (IP) 100 μg/Kg dose of LPS (SAC/LPS100), and the other a 400 μg/Kg dose (SAC/LPS400). A third group received a saccharin/LPS (100 μg/Kg) pairing; however, the animals were allowed water, but not saccharin, on the test day. This group was implemented so as to control for any effect of the saccharin/LPS pairing on plasma iron concentration.

On the initial day of the experiment, rats were placed on a water deprivation regime. This schedule allowed the animals 15 min of drinking at 09:00 h each day. This time period was the only opportunity the animals had to consume fluid. A two-bottle system was employed, with a bottle presented on both the right-hand and left-hand side of the cage. For the water training section of the experiment, both bottles contained tapwater. On the conditioning and test days, however, one bottle contained water, whereas the other contained the relevant CS. This allowed particular experimental groups the option of drinking either saccharin or water on these days.

Seven days following the commencement of water deprivation, the conditioning day procedure was employed. This involved supplying each rat with the relevant CS during the allotted drinking time. Immediately after this period, each rat received an IP injection of the relevant UCS in a dose volume of 1.0 ml. After a further 7 days, animals were again presented with the appropriate CS during the drinking session. Rats were subsequently administered an IP injection of 0.9% pyrogen-free saline.

Seven hours following CS representation, rats were decapitated and trunk blood was collected in microcentrifuge tubes. Serum samples were then centrifuged at 3000 rpm for 5 min. Plasma was subsequently pipetted into aliquots and immediately stored at −80°C until the analysis of plasma iron concentration. To determine the plasma concentration of iron, 0.2 ml of serum and 0.2 ml of distilled water (control) were each pipetted into a separate microabsorption cell. Subsequently, 1 ml of buffer solution was added to each cell, and the cell centrifuged at 2000 rpm for 1 min. This allowed iron to be released from transferrin by guanidine hydrochloride, and consequently reduced to Fe^{2+} by hydroxylamine. Chromogen was then added to the cells, centrifuged at 2000 rpm for 3 min, and incubated for 20 min at +25°C. This allowed ferrozine to mix with bivalent iron, to form a red complex. The optical density of the coloured complex was measured using a spectrophotometer, and plasma iron determined by comparison with standard curves (Roche Diagnostics).

Statistical Procedure

Saccharin consumption data were analysed using two one-way (group) analyses of variance. Plasma iron was analysed using a one-way (group) analysis of variance. Post hoc analy-

ses were completed using Student's t-test, including Bonferroni α-adjustments to eliminate experimenter-wise error. Pearsons correlation was also completed on the combined saccharin and iron data.

RESULTS

The mean saccharin consumption for the two SAC/LPS groups and the SAC/SAL group is displayed in Fig. 1.

On the conditioning day, no significant differences were found among the three groups [$F(2, 19) = 2.59$, $p > 0.05$]. However, saccharin consumption on the test day was significantly different among the groups [$F(2, 19) = 4.09$, $p < 0.01$]. Post hoc simple effects analysis revealed that on the test day, the SAC/LPS400 consumed less saccharin than the SAC/LPS100 and SAC/SAL groups ($p < 0.01$). The SAC/LPS100 group displayed lower saccharin consumption than the SAC/SAL group; however, this difference was not significant.

The mean levels of plasma iron on the test day are displayed in Fig. 2, which shows that the two SAC/LPS groups experienced reduced levels of plasma iron on the test day. The SAC/LPS400 animals had a slightly lower level of iron than the SAC/LPS100 group. A one-way analysis of variance revealed a significant difference in plasma iron levels among the groups [$F(5, 40) = 16.873$, $p < 0.001$]. Post hoc analysis demonstrated that the SAC/LPS100 and SAC/LPS400 groups did not significantly differ in levels of plasma iron. The remaining four control groups did not display significant differences among them. However, both of the SAC/LPS groups experienced lower plasma iron levels than the SAC/SAL ($p < 0.001$), WAT/LPS ($p < 0.001$), and WAT/SAL ($p < 0.001$) animals.

Because of the significant decrease in plasma iron in the SAC/LPS100 animals, but concomitant nonsignificant saccharin consumption reduction, a correlation was performed between the saccharin and iron data for the SAC/LPS100 and SAC/SAL groups. This revealed no significant relationship between saccharin consumption and concentration of plasma iron ($r = 0.34$, $p > 0.05$).

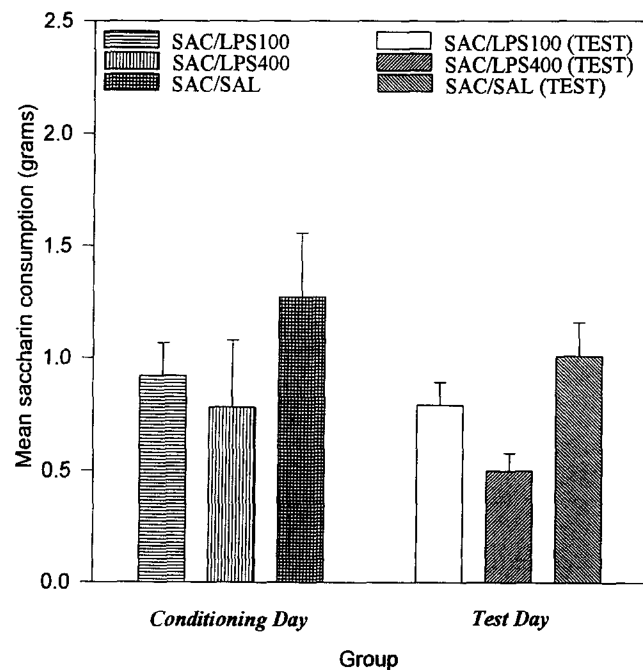

FIG. 1. Mean saccharin consumption on the conditioning day (= CS-UCS pairing) and test day (CS representation).

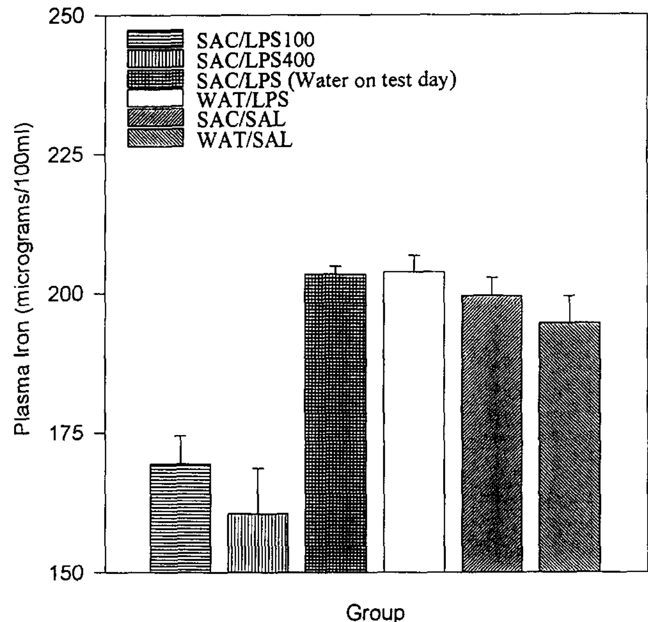

FIG. 2. Mean levels of plasma iron on the test day.

DISCUSSION

The present results support those previously obtained in this laboratory, demonstrating that acute-phase immune responses are conditionable using CTA (2,3,9,10). These results are in accordance with the well-documented decrease in plasma iron concentration in response to bacterial infection (1,4,6,8,11,12,15,16,18,20,28,35,37,39,40). The results of this study did not demonstrate a dose-dependent effect of plasma iron conditioning. Rats injected with 400 μg/Kg of LPS as the UCS developed a conditioned decrease in plasma iron not significantly different from those animals infused with 100 μg/Kg. This result appears contrary to the results of Kampschmidt and Upchurch (16), who showed a dose-dependent decrease of plasma iron in response to bacterial endotoxin. It is possible that the conditioned response may have a hypoferremic nadir, abolishing any effects of differential LPS dosages. The conditioned levels of hypoferremia are of lower magnitude than those reported during the acute response to endotoxin (16). This result is concordant with the conditioning of other acute-phase responses, in which the test day response is typically lower in amplitude than the conditioning day response (2,3,9,10). The nonsignificant difference between the two SAC/LPS groups may be due to this phenomenon. The converging plasma iron levels of the two experimental groups on the test day may be the result of the conditioned effects being proportionally lower for the SAC/LPS400 group than the SAC/LPS100 animals. In addition, the nonsignificant difference between these groups may be due to a ceiling effect of LPS on plasma iron. Dose-dependent studies of endotoxin have shown a larger decrease with increasing endotoxin administration; however, this result was demonstrated with a maximum endotoxin dose of 100 μg/Kg (16). Thus, a 400-μg

infusion of LPS may not decrease plasma iron more than a 100-μg dose, possibly because of there being an optimum plasma iron level for the host organism to produce so as to combat infection. An interesting result is that the conditioned hypoferremia in the SAC/LPS100 group was not associated with significant taste aversion. Data analysis revealed no relationship between the amount of saccharin consumed and levels of plasma iron. This is the first report of a dissociation between CTA and conditioned acute-phase responses from this laboratory. It appears that CTA may not be a prerequisite as such for conditioned immunomodulation.

Endotoxin is thought to generate acute-phase responses via the production of interleukin 1 (IL-1), and the subsequent synthesis of prostaglandins of the E series, which act on both peripheral organs and the CNS (25). Similar to other acute-phase responses, the fall in plasma iron is largely mediated through the action of IL-1 (17,19,42). However, unlike sleep and fever, but analogous to anorexia, the action of IL-1 in reducing plasma iron levels may occur at both a peripheral and central site (30–32). However, unlike other acute-phase responses that have been demonstrated to be conditionable, hypoferremia appears not to be modulated by prostaglandin E_2 within the CNS (31); nonetheless, peripheral arachidonic acid metabolites may activate the reduction of plasma iron.

The cellular mechanisms responsible for hypoferremia in response to infection has not been fully elucidated. It has been posited that the decrease in plasma iron in response to endotoxin is due to an impairment in iron release from certain tissues, particularly the liver, lung, and gut (5,26). Investigations of the hepatic parancymal cell response to endotoxin, the main site of iron storage within the liver, have shown no alteration in release or pickup of iron (27), and conversely, increased iron uptake from transferrin (34). In addition, IL-1 was shown to have no effect on iron release from the mononuclear phagocyte system (38). Thus, these equivocal results suggest that the cellular mechanism involved in hypoferremia, and the conditioning of this state, may be at least partly mediated by molecules other than IL-1, such as prostaglandins.

The results of this report extend the knowledge of conditioning parameters of the immune system. The present findings demonstrate that in addition to neurophysiologic (2,3,10) and behavioral (9) factors being conditionable using CTA, the metabolic response of the host to infection can be manipulated via behavioral modification. This suggests that via cognitive pathways, the orchestra of acute-phase responses may be able to be altered simultaneously, promoting an optimum environment for pathogen elimination.

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REFERENCES

1. Blatteis, C. M.; Mashburn, T. A; Ahokas, R. A. Fever and trace metal changes in endotoxin-challenged neonates. Pflugers Arch. 389:177–179; 1981.
2. Bull, D. F.; Brown, R.; King, M. G.; Husband, A. J. Modulation of body temperature through taste aversion conditioning. Physiol. Behav. 49:1229–1233; 1991.
3. Bull, D. F.; Exton, M. S.; Husband, A. J. The acute phase immune response: Lipopolysaccharide induced fever and sleep alterations are not simultaneously conditionable in the rat during the inactive (light) phase. Physiol. Behav. In press.
4. Butler, T. J.; Curtis, M. J. The effect of *Escherichia coli* endotoxin on the plasma iron concentration in the domestic fowl. Res. Vet. Sci. 15:267–269; 1973.
5. Cortell, S.; Conrad, M. E. Effect of endotoxin on iron absorbtion. Am. J. Physiol. 213:43–47; 1967.
6. Depelchin, B. O.; Bloden, S.; Hooremans, M.; Noirfalise, A.; Ansay, M. Clinical and experimental modifications of plasma iron and zinc concentrations in cattle. Vet. Rec. 116:519–521; 1985.
7. Elin, R. J.; Wolff, S. M. The role of iron in nonspecific resistance to infection induced by endotoxin. J. Immunol. 112:737–745; 1974.
8. Emody, L.; Ralovich, B.; Barna, K.; Brasch, G.; Ternak, G. Physiological effect of orally administered endotoxin in man. J. Hygiene Epidemiol. Microbiol. 18:454–458; 1974.
9. Exton, M. S.; Bull, D. F.; King, M. G.; Husband, A. J. Behavioral conditioning of endotoxin induced anorexia. Physiol. Behav. (in press).
10. Exton, M. S.; Bull, D. F.; King, M. G.; Husband, A. J. Modification of body temperature and sleep state using behavioral conditioning. Physiol. Behav. (in press).
11. Greiger, T. A.; Kluger, M. J. Fever and survival: The role of serum iron. J. Physiol. 279:187–196; 1978.
12. Gutteberg, T. J.; Rokke, O.; Anderson, O.; Jorgensen, T. Early fall of circulating iron and rapid rise of lactoferrin in septicemia and endotoxemia: An early defence mechanism. Scand. J. Infect. Dis. 21:709–715; 1989.
13. Jones, R. L.; Peterson, C. M.; Grady, R. W.; Kumbaraci, T.; Cerami, A.; Graziano, J. H. Effects of iron chelators and iron overload on Salmonella infection. Nature 267:63–65; 1977.
14. Joo, I. Virulence-enhencing effect of ferric ammonium citrate on *Vibrio cholerae*. Prog. Drug Res. 19:546–553; 1975.
15. Kampschmidt, R. F.; Schultz, G. A. Hypoferremia in rats following injection of bacterial endotoxin. Proc. Soc. Exp. Biol. Med. 106:870–871; 1961.
16. Kampschmidt, R. F.; Upchurch, H. F. Effects of bacterial endotoxin on plasma iron. Pro. Soc. Exp. Biol. Med. 110:191–193; 1962.
17. Kampschmidt, R. F.; Upchurch, H. F. A comparison of the effects of rabbit endogenous pyrogen on the body temperature of the rabbit and lowering of plasma iron in the rat. Proc. Soc. Exp. Biol. Med. 133:128–130; 1970.
18. Kampschmidt, R. F.; Upchurch, H. F.; Eddington, C. L. Hypoferremia produced by plasma from endotoxin treated rats. Pro. Soc. Exp. Biol. Med. 132:817–820; 1969.
19. Kampschmidt, R. F.; Upchurch, H. F.; Pulliam, L. A. Investigations on the mode of action of endogenous mediator. Proc. Soc. Exp. Biol. Med. 143:279–283; 1973.
20. Kluger, M. J.; Rothenberg, B. A. Fever and reduced iron: Their interaction as a host defence response to infection. Science 203: 374–376; 1979.
21. Kluger, M. J.; Rothenberg, B. A. Fever, trace metals, and disease. In: Lipton, J. M., ed. Fever. New York: Raven Press; 1980.
22. Kluger, M. J. Fever: The role of pyrogens and cryogens. Physiol. Rev. 71:93–127; 1991.
23. Kochan, I. The role of iron in bacterial infections, with special consideration of host-tubercle bacillus interaction. Curr. Top. Microbiol. Immun. 60:1–30; 1973.
24. Kochan, I.; Wasynczuk, J.; McCabe, M. A. Effects of injected iron and sideophores on infections in normal and immune mice. Infect. Immun. 22:560–567; 1978.
25. Kushner, I. The acute phase response: An overview. Methods Enzymol. 163:373–383; 1988.
26. Lewis, M. Tissue distribution of hemoglobin iron in endotoxin-induced hypoferremia. J. Retic. Soc. 11:198–205; 1972.
27. Lewis, M. *In vitro* investigations in endotoxin-induced hypoferremia. J. Retic. Soc. 14:301–308; 1973.
28. Mashburn, T. A. Jr.; Llanos-Q.; J.; Hunter, W. S.; Ahokas, R. A.; Blatteis, C. M. Differential acute-phase resonses in febrile

and cold- and heat-exposed rabbits. Pflugers Arch. 402:157–161; 1984.

29. Miles, A. A.; Pillow, J.; Khimji, P. L. The action of iron on Klebsiella infection on the skin of the guinea pig and its relation to the decisive period in primary infective lesions. Br. J. Exp. Pathol. 57:217–242; 1976.
30. Morimoto, A.; Murakami, N.; Myogin, T.; Takada, M.; Teshirogi, S.; Watanabe, T. Separate mechanisms inside and outside the blood–brain barrier inducing metabolic changes in febrile rabbits. J. Physiol. 392:627–649; 1987.
31. Morimoto, A.; Murakami, N.; Watanabe, T. Is the central arachidonic acid cascade system involved in the development of acute-phase response in rabbits? J. Physiol.397: 281–289; 1988.
32. Morimoto, A.; Sakata, Y.; Watanabe, T.; Murakami, N. Characteristics of fever and acute-phase response induced in rabbits by IL-1 and TNF. Am. J. Physiol. 256:R35–R41; 1989.
33. Payne, S. M.; Finkelstein, R. A. Pathogenesis and immunology of experimental gonococcal infection: Role of iron in virilence. Infect. Immun. 12:1313–1318; 1975.
34. Potter, B. J.; Blades, B.; McHugh, T. A.; Nunes, R. M.; Beloqui, O.; Slott, P. A.; Rand, J. H. Effects of endotoxin on iron uptake by the hepatocyte. Am. J. Physiol. 257:G524–G531; 1989.
35. Tegowska, E.; Wasilewska, E. Effect of ambient temperature and *E. coli* endotoxin upon the plasma iron level in wild house mice in winter season. J. Comp. Physiol. B. 162:327–330; 1992.
36. Tsukano, H.; Yamamoto, M.; Wake, A. The effect of an iron drug on the growth of plague microorganisms *in vitro* and in guinea pig skin. Jpn. J. Med. Sci. Biol. 25:85–93; 1972.
37. Tufft, L. S.; Nockels, C. F.; Fettman, M. J. Effects of *Escherichia coli* on iron, copper, and zinc metabolism in chicks. Avian Dis. 32:779–786; 1988.
38. Uchida, T.; Yamagiwa, A.; Nakamura, K. The effect of interleukin-1 on iron metabolism in rats. Eur. J. Haematol. 46:1–5; 1991.
39. Van Miert, A. S. J. P. A. M.; Van Duin, C. T. M.; Wensing, T. Fever and changes in plasma zinc and iron concentrations in the goat. The effects of interferon inducers and recombinant IFN-α_{2a}. J. Comp. Pathol. 103:289–300; 1990.
40. Verheijden, J. H. M.; van Miert, A. S. J. P. A. M.; Schotman, A. J. H.; van Duin, C. T. M. Plasma zinc and iron concentrations as measurements for evaluating the influence of endotoxin-neutralizing agents in *Escherichia coli* endotoxin-induced mastitus. Am. J. Vet. Res. 43:724–728; 1982.
41. Weinberg, E. D. Nutritional immunity: Hosts attempt to withold iron from microbial invaders. JAMA 231:39–41; 1975.
42. Yamashiro, O.; Morimoto, A.; Sakata, Y.; Watnabe, T.; Murakami, N. Febrile and metabolic tolerance to endotoxin and human recombinant interleukin-1β in rabbits. Am. J. Physiol. 264: R1180–R1185; 1993.