INFECTION AND IMMUNITY, Sept. 1980, p. 886-891
0019-9567/80/09-0886/06$02.00/0

Vol. 29, No. 3

Iron-Controlled Infection with *Neisseria meningitidis* in Mice

BRUCE E. HOLBEIN

Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B4

An iron-controlled infection was obtained after the intraperitoneal injection of *Neisseria meningitidis* strain M1011 into normal mice. The infection progressed rapidly but then disappeared in concert with the disappearance of plasma transferrin iron. Parenteral iron dextran enhanced and prolonged the infection in mice at dosages above 15 mg of Fe per kg. Studies on the distribution of iron dextran within the physiological iron pools and the importance of timing with the iron dextran addition indicated that high serum iron, available early during infection, was necessary to promote infection. High levels of iron in the reticuloendothelial system did not stimulate infection. A working hypothesis to explain the roles of iron in infection was developed: *N. meningitidis* obtains iron for growth from the transferrin pool, and iron dextran maintains transferrin iron levels during infection.

A growing body of evidence indicates that iron availability plays a key role in pathogenesis by bacteria (6, 24). Two major aspects accounting for this importance of iron are the absolute requirements that bacteria have for iron and the relative unavailability of iron for microbial growth within the body.

Body iron stores are associated with various proteins which have very high affinities for iron. Serum transferrin, for example, which is only approximately 30% saturated in humans but has an association constant of approximately 10^{36}, results in a free serum iron concentration of about 10^{-18} M (6). This level of iron is far below that required for bacterial growth (24), and the in vitro bacteriostatic and bactericidal activities of sera have been related to their iron contents (8, 14, 15, 18).

Although iron may become more available during disease from hemorrhage, lysis of erythrocytes, or tissue necrosis, the initial low level of free iron suggests that a bacterial pathogen must employ an iron acquisition mechanism(s) capable of competing with host iron-binding proteins at least during the establishment of infection. *Salmonella typhimurium* (17) and, recently, *Neisseria meningitidis* (2) have been found capable of removing iron from transferrin in vitro.

In addition to the initial low level of available iron, a host responds to infection by reducing iron levels in the transferrin pool and increasing levels of storage iron (5, 6, 24). This iron sequestration response can be regarded as a form of nutritional immunity (24) and has been observed after the injection of live organisms or sham infectious materials (5-8, 24). Conditions of abnormally high iron status appear to render humans more susceptible to disease, and iron administration to other animals has been shown to greatly enhance their susceptibilities to experimental bacterial infections (6, 11, 12, 15, 24).

The problem with experiments involving iron additions to animals is in defining the exact role(s) of the added iron. It appears that a major role is iron supply to bacteria (6, 24), but the possibility that added iron renders animals more susceptible by its interference with other defense mechanisms cannot be ruled out. Iron has been shown to interfere with the normal functioning of phagocytic cells (13, 25). Few studies have been made of the distribution of injected iron or related the presence of additional iron in a given physiological pool to the enhancement of infection. A study of normal iron levels in relation to infection could also yield important information on the role of iron in pathogenesis.

My co-workers and I recently reported a model for the study of the virulence of *N. meningitidis* in which injected iron dextran resulted in up to a 10^9-fold decrease in 50% lethal doses for some strains, although not affecting the 50% lethal doses for others (12). We had observed pathological evidence for infection in mice which had not received iron dextran but had been injected with virulent strains, i.e., evidence for nonfatal infection without iron addition. In this report, infection with a virulent strain of *N. meningitidis* was examined in mice without iron addition. It was found that in mice of normal iron status rapid infection occurred but disappeared in concert with transferrin iron. The timing of the iron dextran addition and its fate within the physiological iron pools were also examined to determine the possible roles of iron dextran in the enhancement of infection. The evidence suggests that serum iron rather than

reticuloendothelial system (RES) iron is important in determining the course of infection.

MATERIALS AND METHODS

Bacterial strain. *N. meningitidis* strain M1011 used for this study was initially isolated from clinical disease by N. Vedros and obtained from C. E. Frasch. Strain M1011 is a serogroup B and carries serotype antigens 2 and 10 (9). It was maintained, cultivated on Columbia blood agar, and examined for its virulence as described previously (12).

Mice. C57 black male mice were given a diet containing 150 mg of Fe per kg (12) to maintain a normal iron balance (22).

Infection studies. Mice were injected intraperitoneally (i.p.) with approximately 10^4 colony-forming units (CFU) of strain M1011, grown on Columbia blood agar, and suspended in 0.5 ml of Neisseria Chemically Defined Medium (GIBCO Diagnostics, Madison, Wis.) at zero time. Mice were examined for symptoms (12), and two mice per group were bled by cardiac puncture into 2.0-ml vacutainer tubes (Becton Dickinson & Co., Rutherford, N.J.) containing 0.1 ml of 3.8% (wt/vol) sodium citrate at intervals over 24 h. Approximately 1.0 ml of blood was obtained by this fatal procedure. Thus, each experiment included enough mice for bleeding and 20 infected mice for monitoring symptoms to 72 h. Blood samples were diluted serially in Neisseria Chemically Defined Medium to determine the CFU per milliliter of blood (12). An additional five mice were bled, and their blood was divided into two separate samples. Plasma obtained from these samples after centrifugation at 12,800 × *g* for 3 min at 22°C was assayed for total iron-binding capacity, unsaturated iron-binding capacity, and transferrin iron by a ^{59}Fe radioassay (Becton, Dickinson & Co.). The effects of added iron on infection were examined by injecting 0.5 ml of iron dextran i.p. immediately before the bacterial injection to achieve dosages of 15, 30, 60, 125, and 250 mg of Fe per kg (12). Mortality from infection was scored at 72 h postinjection.

Fate of injected iron dextran. Mice were injected with iron dextran at a dosage of 250 mg of Fe per kg and bled by cardiac puncture into 2.0-ml vacutainer tubes containing no anticoagulants at intervals between 0 and 72 h postinjection. Sera from five mice were pooled and analyzed for total serum iron by a modification of the method of Olson and Hamblin (19); 0.5 ml of serum was mixed with 0.5 ml of 20% (wt/vol) trichloroacetic acid and heated at 90°C for 15 min, and supernatant fractions obtained by centrifugation at 12,800 × *g* for 5 min at 22°C were assayed for iron by atomic absorption spectrophotometry (12). This procedure was used to measure both transferrin iron and any free iron dextran in sera. Transferrin iron was measured specifically by a ^{59}Fe radioassay, a procedure which was not interfered with by free iron dextran at 500 μg of Fe per ml (unpublished data). Iron storage, at various times after iron injection, was assessed by flame-absorption spectrophotometric iron analyses of the livers and spleens of 20 mice after digestion in tetramethylammonium hydroxide (10). Tissue samples were also fixed, embedded, sectioned, stained (12), and poststained for iron (4). Iron deposits were compared with control tissues by light microscopy. The effects of the timing of the iron dextran addition were examined by injecting mice with a dosage of 250 mg of Fe per kg at various times before and after the bacterial injection.

RESULTS

Iron-limited infection in normal mice. Infection in normal mice was investigated first to determine if *N. meningitidis* could establish infection without added iron. Strain M1011 was used for these experiments as it displayed a high virulence (50% lethal dose < 2 CFU) in mice given iron dextran (12). Strain M1011 produced a rapid bacteremia after an i.p. injection into normal mice (Fig. 1). High numbers of bacteria were evident in the blood at 6 h postinjection, and symptoms of infection, including inactivity and lachrymation, were also present in all infected mice. Infection disappeared rapidly after 9 h, and bacteria were never recovered from the blood at 18 h postinjection. The disappearance of symptoms correlated with the loss of bacteremia; all the mice appeared healthy at 18 to 24 h and remained healthy until the conclusion of the experiments at 72 h. An examination of the status of the transferrin iron pool during the infection yielded valuable information. The unsaturated iron-binding capacity increased immediately and continuously after the injection of bacteria. The total amount of plasma transferrin (total iron-binding capacity) remained

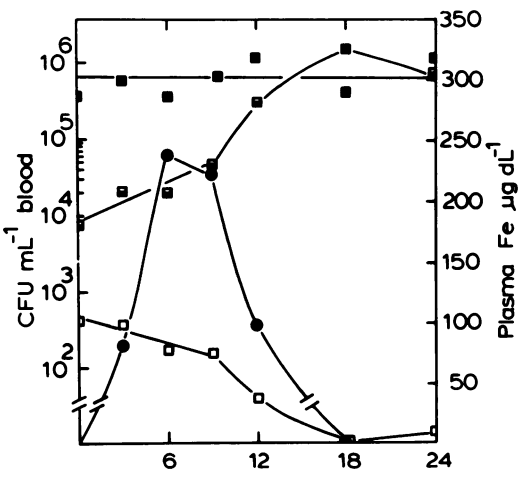

FIG. 1. *Iron-controlled infection with N. meningitidis in normal mice. Normal mice were injected i.p. with approximately 10^4 CFU of N. meningitidis strain M1011, and bacteria in blood (●) were monitored over a 24-h period. Plasma was analyzed for total iron-binding capacity (■), unsaturated iron-binding capacity (▬), and transferrin iron (□). Values represent average data from two separate experiments.*

constant and, thus, transferrin iron levels fell continuously during infection. This fall in the transferrin iron level appeared to be biphasic, i.e., a rapid fall occurring after 9 h postinjection. Virtually no transferrin iron could be detected in plasma obtained at 18 or 24 h after the injection of bacteria. The rapid decrease in the numbers of bacteria in the blood paralleled the rapid decrease in transferrin iron levels.

Stimulation of infection by parenteral iron dextran. Studies with normal mice indicated that infection may have been controlled due to iron limitation and, therefore, iron addition was investigated to see if it would stimulate infection. Parenteral iron dextran at dosages above 15 mg of Fe per kg prolonged and greatly enhanced infection (Fig. 2). High numbers of bacteria were found beyond 18 h postinjection in the blood of mice given iron at ≥30 mg of Fe per kg. However, an identical course of infection, as seen in normal mice, was observed with an iron dosage of 15 mg of Fe per kg. Once again, symptoms of infection correlated with the levels of bacteremia.

Fatal infection, however, did not occur until dosages of ≥125 mg of Fe per kg were given. Twenty-five percent of the mice given 125 mg of Fe per kg were dead by 72 h postinjection, but all mice given 250 mg of Fe per kg died of infection; this was a surprising result in view of the similar courses of infection with these two iron dosages up until 24 h postinjection. Mice given 60 or 30 mg of Fe per kg were fully recovered and healthy at 72 h postinjection. Thus, these results indicated that the level of bacteremia at 24 h postinjection was related to the amount of iron added but did not correlate with the outcome of infection.

Fate of parenteral iron dextran and the importance of timing with its administration. The fate of iron dextran after injection was investigated to study the time course of its distribution within the physiological iron pools so that the location of extra iron could be related to its stimulation of infection. Iron dextran entered the circulatory system rapidly after an i.p. injection, with peak levels of total serum iron occurring at about 2 h postinjection (Fig. 3A). Total serum iron levels then rapidly declined to preinjection levels by about 48 h postinjection. Elevated iron levels were detected in the liver and spleen at 6 h and peaked at about 48 h postinjection (Fig. 3A). Thus, these data indicated that iron dextran was rapidly removed from the circulation by the RES. The location of iron in the RES was confirmed by a histochemical examination of livers and spleens, where iron deposits could be observed in the liver sinusoids and the white pulp of the spleen

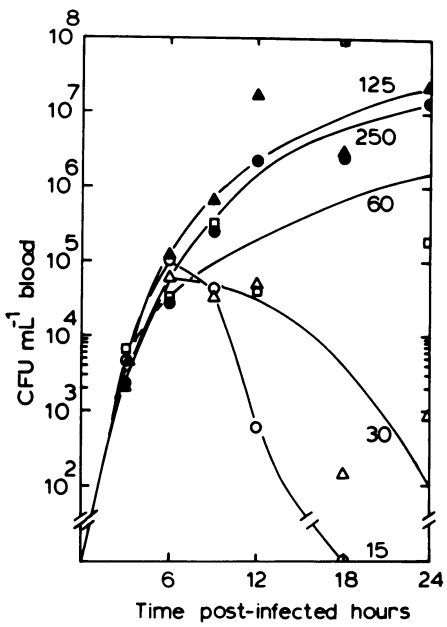

FIG. 2. *Stimulation of infection by parenteral iron dextran. Mice were injected i.p. with iron dextran at dosages of 15* (○), *30* (△), *60* (□), *125* (▲), *and 250* (●) *mg of Fe per kg and then infected with approximately 10^4 CFU of N. meningitidis strain M1011. Bacteremia was monitored for 24 h. Values represent average data from two separate experiments.*

(data not shown). Iron entered the transferrin pool soon after injection, and the levels increased at least until 6 h postinjection (Fig. 3B). Transferrin levels remained constant over the 72-h observation period, indicating that increased transferrin synthesis did not result from the iron injection. The slight delay in the entry of iron into the transferrin pool and the kinetics of uptake of iron dextran into the RES provided evidence in support of the hypothesis of others (16, 23) that an iron-containing colloid, such as iron dextran, is taken into the RES and processed before the release of its iron to the transferrin pool.

Two important periods of time were described by the data of Fig. 3. Early after the iron injection (≤6 h), high serum iron, as free iron dextran and transferrin iron, but low RES iron existed. Later after the injection (≥24 h), low serum iron but high RES iron existed. This information was used to specifically examine the importance of timing (i.e., iron location) in the enhancement of infection by iron (Table 1). Iron dextran given 24 or 48 h before the injection of bacteria did not promote fatal infection in mice. Thus, high RES iron was not involved in promoting infection. Iron dextran given at up to 6 h after the

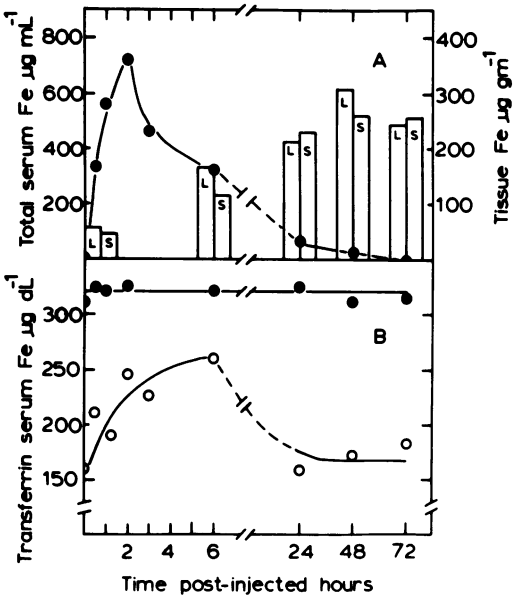

FIG. 3. *Distribution of parenteral iron dextran. Mice were injected i.p. with iron dextran at a dosage of 250 mg of Fe per kg, and at intervals sera, livers, and spleens were obtained for iron analyses. (A) Total serum iron (●) and iron in livers (L) and spleens (S). Values at time zero are for uninjected mice. Total serum iron values represent averages from two separate experiments, and values for tissues represent averages for 20 samples each. (B) Total iron-binding capacity (●) and transferrin iron (○). Values represent average data from two separate experiments.*

injection of bacteria did promote fatal infection, indicating that high serum iron was necessary to stimulate infection.

DISCUSSION

The rapid bacteremia that occurred after the i.p. injection on *N. meningitidis* strain M1011 into normal mice revealed some important aspects of the virulence of this organism. The bacterium must have escaped from phagocytes in the peritoneal cavity, possessed the ability to invade the bloodstream from the peritoneal cavity, and obtained sufficient iron for its rapid growth. Infection in normal mice progressed for only a few hours and then rapidly disappeared, i.e., the infection was controlled. Control of the infection correlated with decreasing iron levels in the transferrin pool. Thus, it appeared reasonable to assume that the infection had been arrested by iron unavailability. Two possibilities exist to explain how low transferrin iron levels may have been achieved during infection. The first is that *N. meningitidis* consumed transferrin iron during growth, but insufficient iron was available for continued growth. This organism has been shown to use human transferrin iron in vitro (2) and, therefore, it may use mouse transferrin iron in vivo. However, the relatively low in vitro iron requirement of *N. meningitidis* in defined media (<100 ng/ml) (1) and the fact that initially approximately 1 µg of mouse transferrin iron per ml was available to the organism raised the second possibility. This second possibility is that *N. meningitidis* used transferrin iron initially but that mice responded to the infection with iron sequestration. The apparent biphasic nature of the disappearance of transferrin iron is consistent with this view. The importance of transferrin iron in infection was revealed by the iron addition. Parenteral iron dextran stimulated infection in a dose-dependent manner, but studies on the distribution of parenteral iron and the importance of timing with its administration indicated that the extra iron had to be in the transferrin pool to promote infection. Presumably, the extra iron overcame the ability of the host iron sequestration mechanisms to lower transferrin iron levels.

The present results provide evidence that an iron overload of the RES does not alter the ability of *N. meningitidis* to establish an infection in mice. Mice given iron 24 h or more before being infected behaved as normal mice. Thus, it cannot be argued that an iron overload of the RES impairs normal defense mechanisms which operate to prevent infection. However, the outcome of infection was different in mice given high levels of parenteral iron. Only mice given the highest levels of iron died. It appears possible

TABLE 1. *Importance of timing of the iron dextran addition in the stimulation of infection with N. meningitidis strain M1011*

Time of iron dextran addition (h)[a]	Symptoms of infection at 24 h after bacterial injection[b]	% Mortality at 72 h after bacterial injection
−48	Slight	0
−24	Moderate to severe	8
0 (control)	Severe	100
3	Severe	100
6	Severe	100
No iron (control)	None	0
No bacteria (control)	None	0

[a] Iron was injected at a dose of 250 mg per kg 48 and 24 h before the injection of approximately 10^4 CFU of strain M1011 and at 0, 3, and 6 h postinjection of bacteria. Values represent average results from two separate experiments performed with groups of 10 to 15 mice.

[b] Symptoms were rated arbitrarily as to intensity as compared with controls.

then that an iron overload of the RES does play a role but only later, after the infection has been established and has progressed. An RES blockade has been shown to augment the generalized Schwartzman reaction of rabbits to meningococcal endotoxin (21). It may be that high levels of iron dextran resulted in an RES blockade and increased the susceptibility of mice to endotoxin elaborated during infection. These possibilities have not been examined.

The results of this study have been incorporated into a working hypothesis to explain the roles of iron in experimental mouse infection (Fig. 4). Transferrin iron is used for the growth of *N. meningitidis* in vivo. Parenteral iron dextran supports the infection by maintaining the transferrin iron pool after first being routed through the RES. The possibility that some iron from iron dextran enters the transferrin pool directly cannot be discounted. However, the slight delay in the entry of iron from iron dextran into the transferrin pool and the evidence obtained with other animals (16, 23) suggest that iron dextran enters the RES first. The bulk of the injected iron dextran is removed to storage by the RES, where it does not influence infection. It is unknown if *N. meningitidis* can obtain iron directly from iron dextran in vivo. There is some uncertainty as to whether this can occur in vitro. Payne and Finkelstein (20) had shown that iron dextran stimulated the growth of *N. meningitidis* on Thayer-Martin medium, but Archibald and DeVoe (3), using a sensitive assay with iron-limited, defined media, did not observe the stimulation of growth by iron dextran.

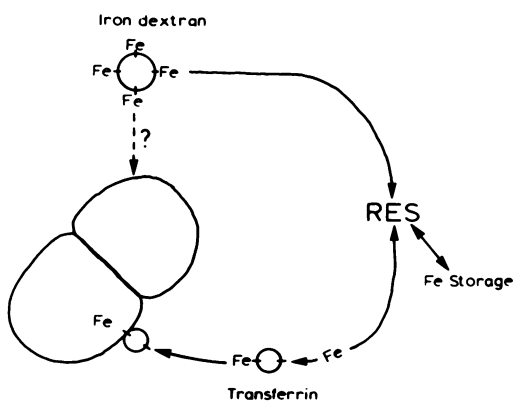

FIG. 4. *Working hypothesis for the roles of iron in N. meningitidis infection in mice. N. meningitidis obtains iron for growth from the transferrin iron pool, which disappears during the infection of normal mice. Iron dextran is taken into the RES, and part of its iron is routed to the transferrin pool, thus promoting infection. The bulk of the iron from iron dextran remains as stored iron in the RES, where it does not stimulate infection. It is unknown if iron dextran is directly available to N. meningitidis in vivo.*

Infection in normal mice provides a useful model for studying the roles of iron and iron sequestration during infection, and ways of manipulating the status of the transferrin iron pool to determine its importance in the establishment of infection are being examined at this laboratory.

ACKNOWLEDGMENTS

The bulk of this study was performed while I was with the Preventive Medicine Section, Defence Research Establishment, Suffield, Ralston, Alberta, Canada, and I wish to thank the establishment and the director of Preventive Medicine for the Canadian Armed Forces for support for this research.

K. W. F. Jericho is thanked for his histological examinations as well as G. C. Likes and M. R. Spence for their excellent technical assistance. I. W. DeVoe is thanked for his valuable criticisms during the preparation of the manuscript.

LITERATURE CITED

1. **Archibald, F. S., and I. W. DeVoe.** 1978. Iron in *Neisseria meningitidis*: minimum requirements, effects of limitation, and characteristics of uptake. J. Bacteriol. **136**:35–48.
2. **Archibald, F. S., and I. W. DeVoe.** 1979. Removal of iron from human transferrin by *Neisseria meningitidis*. FEMS Lett. **6**:159–162.
3. **Archibald, F. S., and I. W. DeVoe.** 1980. Iron acquisition by *Neisseria meningitidis* in vitro. Infect. Immun. **27**:322–334.
4. **Barka, T., and P. J. Anderson.** 1963. Histochemistry, p. 174. Harper & Row, Publishers, New York.
5. **Beisel, W. R.** 1977. Magnitude of the host nutritional responses to infection. Am. J. Clin. Nutr. **30**:1236–1247.
6. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1978. Role of iron in bacterial infection. Curr. Top. Microbiol. Immunol. **80**:1–35.
7. **Edwards, C. Q., A. Deiss, B. C. Cole, and J. R. Ward.** 1975. Hematologic changes in chronic arthritis of mice induced by *Mycoplasma arthritidis*. Proc. Soc. Exp. Biol. Med. **150**:664–668.
8. **Elin, R. J., and S. M. Wolff.** 1974. The role of iron in nonspecific resistance to infection induced by endotoxin. J. Immunol. **112**:737–745.
9. **Frasch, C. E., and S. S. Chapman.** 1973. Classification of *Neisseria meningitidis* group B into distinct serotypes. III. Application of a new bactericidal-inhibition technique to distribution of serotypes among cases and carriers. J. Infect. Dis. **127**:149–154.
10. **Gross, S. B., and E. S. Parkinson.** 1974. Analyses of metals in human tissues using base (TMAH) digests and graphite furnace atomic absorption spectrophotometry. Interface **3**:10.
11. **Henderson, L. C., S. Kadis, and W. L. Chapman, Jr.** 1978. Influence of iron on *Corynebacterium renale*-induced pyelonephritis in a rat experimental model. Infect. Immun. **21**:540–545.
12. **Holbein, B. E., K. W. F. Jericho, and G. C. Likes.** 1979. *Neisseria meningitidis* infection in mice: influence of iron, variations in virulence among strains, and pathology. Infect. Immun. **24**:545–551.
13. **Kaplan, S. S., P. G. Quie, and R. E. Basford.** 1975. Effect of iron on leukocyte function: inactivation of H_2O_2 by iron. Infect. Immun. **12**:303–308.
14. **Kochan, I., J. T. Kvach, and T. I. Wiles.** 1977. Virulence-associated acquisition of iron in mammalian se-

rum by *Escherichia coli*. J. Infect. Dis. **135**:623–632.
15. **Kochan, I., J. Wasynczuk, and M. A. McCabe.** 1978. Effects of injected iron and siderophores on infections in normal and immune mice. Infect. Immun. **22**:560–567.
16. **Kornfeld, S., B. Chipman, and E. B. Brown.** 1969. Intracellular catabolism of hemoglobin and iron dextran by the rat liver. J. Lab. Clin. Med. **73**:181–193.
17. **Kvach, J. T., T. I. Wiles, M. W. Mellencamp, and I. Kochan.** 1977. Use of transferrin-iron-enterobactin complexes as the source of iron by serum-exposed bacteria. Infect. Immun. **18**:439–445.
18. **Norrod, P., and R. P. Williams.** 1978. Effects of iron and culture filtrates on killing of *Neisseria gonorrhoeae* by normal human serum. Infect. Immun. **21**:918–924.
19. **Olson, A. D., and W. B. Hamblin.** 1969. A new method for serum iron and total iron-binding capacity by atomic absorption spectrophotometry. Clin. Chem. **15**:438–444.
20. **Payne, S. M., and R. A. Finkelstein.** 1977. Imferon agar: improved medium for isolation of pathogenic *Neisseria*. J. Clin. Microbiol. **6**:293–297.
21. **Smith, R. T., L. Thomas, and R. A. Good.** 1953. Generalized Schwartzman reaction. V. Intravenous injection of colloidal iron or carbon on response of rabbits to meningococcal toxin. Proc. Soc. Exp. Biol. Med. **82**: 712–715.
22. **Sorbie, J., and L. S. Valberg.** 1974. Iron balance in the mouse. Lab. Anim. Sci. **24**:900–904.
23. **Thoren-tolling, K., and L. Jonsson.** 1977. Cellular distribution of orally and intramuscularly administered iron dextran in newborn piglets. Can. J. Comp. Med. **41**:318–325.
24. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
25. **Weinberg, J. B., and J. B. Hibbs.** 1977. Endocytosis of red blood cells or haemoglobin by activated macrophages inhibits their tumoricidal effect. Nature (London) **269**:245–247.