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Mechanisms of Pathogenesis in *Listeria monocytogenes* Infection

I. Influence of Iron[1]

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ABSTRACT

SWORD, C. P. (The University of Kansas, Lawrence). Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. J. Bacteriol. **92**: 536–542. 1966.—The effects of ferric and ferrous iron as well as other cations on *Listeria* infection in mice were studied. Iron compounds caused a reduction in the LD_{50} dose of *Listeria*, and, when added to a synthetic medium, proved stimulatory for in vitro growth of the organism. Bacterial counts on spleen and liver tissue from iron-treated mice showed that iron injections caused more rapid growth of bacteria and resulted in higher numbers of organisms in the tissue. The reticuloendothelial system did not appear to be impaired by this treatment. Immunized animals were not affected by iron treatment during challenge. Mice with experimentally induced hemolytic anemia showed increased susceptibility to listeriosis, whereas those treated with Desferal, a specific iron-chelating agent, appeared more resistant. Iron proved stimulatory for the avirulent strain, 9037–7, and resulted in an LD_{50} of 1.3×10^4 organisms in iron-treated animals. Growth of *L. monocytogenes* and mortality from experimental infection appeared to be correlated with availability of iron to the bacteria. The results suggest that host iron metabolism may play a part in the onset and progress of *Listeria* infections.

Evidence which suggests possible participation of host and microbial iron metabolism in the pathogenesis of microbial diseases has accumulated. Conalbumin, a constituent of egg white, was shown by Schade and Caroline (23) to inhibit in vitro growth of many pathogenic and non-pathogenic iron-dependent bacteria by chelating Fe^{+++}. A similar effect was demonstrated for the $\beta 2$ iron-binding serum protein, transferrin (22, 24). Schade (22) showed that growth of *Staphylococcus aureus*, pigmentation, catalase content, and certain other metabolic activities were all influenced by the availability of iron during growth. Jackson and Burrows (11) found that nonpigmented mutants of *Pasteurella pestis* with reduced mouse virulence and inability to proliferate freely in vivo could be restored to full virulence if sublethal amounts of iron were injected with the organisms. This effect appeared specific for iron. Martin et al. (16) showed that prior or concomitant administration of iron salts enhanced the virulence of strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in rats and mice. Injection of small doses of purified iron-free transferrin caused a slight degree of protection against acute infections, but could not suppress iron-enhanced infection with these organisms. It has also been shown that accumulation of iron in cells of the reticuloendothelial system (RES) occurs subsequent to hemolytic phenomena, injection of iron, and during some infectious diseases (28).

The appearance of increased amounts of haptoglobin and transferrin in the serum of mice during the acute stage of experimental infection with *Listeria monocytogenes* (27) lead us to investigate the possibility that iron may be involved in the pathogenesis of listeriosis. The present investigation deals with the effect of injected iron compounds on mouse virulence of *L. monocytogenes*, the effect of iron on in vitro growth of *L. monocytogenes*, and the effect of experimental alterations in host iron metabolism on in vivo growth and mouse virulence of *Listeria*.

[1] A preliminary report of these data was presented at the 65th Annual Meeting of the American Society for Microbiology, Atlantic City, N.J., 25–29 April 1965.

MATERIALS AND METHODS

Bacteria. Virulent *L. monocytogenes* strains A4413, JHH, and 34S, as well as the avirulent rough strain 9037-7, were employed. Strains A4413, JHH, and 9037-7 were obtained from the U.S. Army Biological Laboratories, Fort Detrick, Md. Strain 34S was a serotype 4b isolate of ovine origin (1). The organisms were maintained on Tryptose Agar (Difco) slants stored at −20 C. Infecting inocula were prepared by growing the organisms in broth containing 2% tryptose and 0.5% NaCl for 24 hr. Suitable dilutions were prepared in the above medium, and the bacteria count of the inoculum was determined by quantitative plating on Tryptose Agar. Inocula employed in testing for in vitro stimulation by iron were grown in the synthetic medium described by Welshimer (29) containing salts, amino acids, and vitamins, and were washed once in deionized distilled water prior to use.

Animals. White female Swiss Webster mice (CD-1 strain, pathogen-free, from the Charles River Farms, North Wilmington, Mass.) weighing 18 to 20 g were used in all experiments. Animals were infected by the intraperitoneal (ip) route in 0.2-ml volumes. Blood for hematocrit determinations was obtained by cardiac puncture. Immunized mice received approximately 10^8 viable organisms of strain A4413 followed in 6 days by 10^6 viable organisms of the same strain. Experiments were performed 14 days after the second injection.

Treatment with iron salts and other cations. The following cations were tested for their effect on virulence: Fe^{+++} (ferric ammonium citrate), Fe^{++} ($FeSO_4$), Mn^{++} ($MnSO_4$), Mg^{++} ($MgSO_4$), Ca^{++} ($CaCl_2$), Na^+ (NaCl). Solutions were prepared to contain 400 μg/ml for each cation and were sterilized by autoclaving at 121 C for 15 min, except Fe^{++}, which was filter-sterilized. Animals received three injections by the ip route, each injection containing 80 μg of the respective cations. The first injection was given 24 hr prior to infection, the second on the day of infection, and the third 24 hr after infection. Injection of 1,000 μg of iron in incomplete Freund's adjuvant by the subcutaneous (sc) route 24 hr prior to infection appeared to produce comparable results. Groups of six mice received each dilution of *Listeria*. The animals were observed for 10 days after infection, and LD_{50} values were determined by the method of Reed and Muench (21). The doses of cations employed caused no deaths in noninfected controls.

Antimouse erythrocyte serum. Rabbit antimouse erythrocyte serum was prepared by injecting a saline suspension of washed pooled mouse erythrocytes by the intravenous, sc, and ip routes at weekly intervals. The titer of hemagglutinins was determined by preparation of twofold serial dilutions of serum in 0.5 ml of saline and adding 0.5 ml of a 1% suspension of mouse erythrocytes to each tube. Titrations were incubated at room temperature for 2 hr, followed by an additional overnight period at 4 C. The highest dilution of serum resulting in macroscopic agglutination was defined as containing 1 hemagglutinating unit (HU). The sera employed in this study contained 320 HU/ml.

Hemolytic anemia. Hemolytic anemia was produced in mice prior to infection by injection of phenylhydrazine and by injection of rabbit antimouse erythrocyte serum as described by Kaye and Hook (13). Hemolytic anemia was produced by ip injection of 2.5 mg of phenylhydrazine hydrochloride in 0.1 ml of distilled water 24 hr prior to infection. Hemolytic anemia was also produced by ip injection of 0.1 ml of antimouse erythrocyte serum diluted in saline to contain 16 HU/dose 24 hr prior to infection. Control groups receiving saline and comparable dilutions of normal rabbit serum in saline were included. Groups of 10 animals per dilution of inoculum were infected, and LD_{50} values were determined by the method of Reed and Muench (21).

Effect of iron on in vitro growth. Bacteria prepared as previously described were inoculated in 0.5-ml volumes containing approximately 10^6 organisms into 50 ml of synthetic medium (29) supplemented with concentrations of iron ranging from 0.1 to 100 μg/ml. Nephelo-Culture flasks (Bellco Glass, Inc., Vineland, N.J.) were employed for these cultures. Cultures were incubated at 37 C. Optical density readings were recorded periodically at 620 mμ on a Bausch & Lomb Spectronic-20 colorimeter. The medium contained the amino acid mixture described by Welshimer (29) rather than the alternate acid-hydrolyzed casein. The basic formula did not contain iron, except as a possible contaminant in the reagents employed. For some experiments, the medium was absorbed with 8-hydroxyquinoline as described by Nicholas (19) to remove iron contamination contributed by the components of the medium. Slightly lower control values were obtained with the absorbed medium.

Bacteria in tissue of nontreated infected and iron-treated infected mice. Groups of mice receiving varying doses of *Listeria* were killed periodically throughout the experiment, and the livers and spleens were aseptically removed. Pooled tissue from five animals was homogenized by grinding with sterile sand and Tryptose Broth (Difco) in a mortar and pestle. Dilutions of the homogenates from each infecting dose were plated on Tryptose Agar, and the colonies were counted after incubation at 37 C for 48 hr to determine the number of bacteria per gram of tissue at different stages of infection.

Circulatory clearance experiments. Groups of five iron-treated and five nontreated mice were injected in the tail vein with approximately 10^8 viable cells of *Listeria* strain A4413 contained in 0.1 ml of saline. Blood samples were collected immediately and 40 min after injection by cutting the tail and collecting 20 μliters of blood in a heparinized micropipette. These were diluted in saline and plated on Tryptose Agar; colony counts were then done to determine relative clearance of *L. monocytogenes* from the circulation in iron-treated and nontreated mice.

Peritoneal clearance experiments. Groups of four iron-treated and four nontreated mice were injected ip with approximately 10^8 viable cells of *Listeria* strain A4413 contained in 0.1 ml of saline. Four iron-treated and four nontreated animals were killed immediately and the same number 40 min after injection. The peritoneum was washed with 2.5 ml of Hank's balanced salt solution. Peritoneal washings were centrifuged at 225 × *g* for 1 min to remove host cells and

debris. The supernatant fluid which contains *Listeria* cells not removed by phagocytosis was diluted in saline and plated on Tryptose Agar; colony counts were done to determine relative clearance of *L. monocytogenes* from the peritoneum in iron-treated and nontreated mice.

Iron chelation. Desferal (desferrioxamine B methane sulfonate; CIBA, Summit, N.J.), a specific iron-chelating agent used for treatment of pathological iron deposition, was employed to deplete mice of iron before and during infection. Desferal was dissolved in iron-free saline and given in 0.2-ml doses by the sc route (three injections per day, 5 mg per injection), starting 3 days prior to infection and continuing 5 days after infection.

Plasma and tissue iron. Plasma iron was determined by the method of Schade et al. (25) on pools of heparinized plasma from the opthalmic venous plexus of 10 to 15 mice. Tissue iron was determined by the method of Schade et al. (25) on livers and spleens prepared by wet washing with HNO_3-$HCLO_4$ according to the method of Ballantine and Burford (3), and was neutralized with NaOH prior to assay.

RESULTS

Effect of iron compounds on virulence. Table 1 shows a comparison of the LD_{50} dose of *Listeria* strain A4413 for mice under various conditions of treatment with cations. Treatment with iron, either Fe^{+++} or Fe^{++}, and manganese reduced the LD_{50} dose. In four repeated experiments with iron-treated and nontreated animals, the mean LD_{50} ($\pm$ SD) was 1.5 ($\pm$0.8) $\times$ 10^2 for Fe^{+++}-treated, 0.5 ($\pm$0.4) $\times$ 10 for Fe^{++}-treated, and 2.9 ($\pm$2.4) $\times$ 10^3 for nontreated mice. Similar results were obtained with strains JHH and 34S. Strain 9037-7, reported by Friedman and Kautter (9) to be avirulent, did not cause death in nontreated mice, but had an LD_{50} of 1.3 $\times$ 10^4 organisms in iron-treated mice.

Hemin was also employed in some preliminary experiments and caused a similar effect; however, the low solubility under physiological conditions caused accumulation of crystalline hemin and probable blockade of the RES when injected in doses containing iron concentrations comparable to those employed for other compounds. Therefore, further tests with hemin were not attempted.

Table 2 shows the result of iron treatment of immunized animals during challenge. The resistance acquired by immunization with living organisms was not overcome as a result of iron treatment.

Effect of iron compounds on in vitro growth of bacteria. A stimulatory effect proportional to iron concentration was noted in synthetic medium supplemented with varying levels of iron (Fig. 1). The results of plate counts were consistent with the turbidimetric results presented. Fe^{+++} (ferric ammonium citrate) and Fe^{++} (ferrous sulfate) caused similar growth curves. Insolubility of $FeSO_4$ in the medium at concentrations above 10 μg/ml of ferrous ion prevented the use of higher Fe^{++} concentrations. Similar results were observed with strains A4413, JHH, 34S, and the avirulent strain 9037-7.

Because of its ability to alter the LD_{50} dose, Mn^{++} was added to the synthetic medium to determine its effect on in vitro growth of *Listeria*. Concentrations of 0.1, 1.0, and 10 μg/ml of Mn^{++} markedly inhibited growth in the medium.

Growth of bacteria in iron-treated mice. Figure 2 shows relative bacteria counts in liver tissue of mice receiving 6.0 $\times$ 10 cells of *Listeria* strain A4413. A similar effect was consistently observed in both liver and spleen tissue of mice receiving different levels of *Listeria* infection ranging from 10^5 to 10 organisms. The organisms appeared to grow more rapidly in both liver and spleen of treated animals, regardless of the infecting dose. The maximal number of organisms occurring in tissue of treated animals receiving comparable infecting doses was consistently greater than that in nontreated infected animals.

Figure 3 shows the results of a similar experi-

TABLE 1. *LD_{50} values of Listeria monocytogenes A4413 for mice treated with ferric, ferrous, and other cations*

Treatment[a]	LD_{50} value[b]
None (control)	7.2 $\times$ 10^3
Fe^{++}($FeSO_4$)	0.6 $\times$ 10
Fe^{+++} (Ferric ammonium citrate)	1.8 $\times$ 10^2
Mn^{++} ($MnSO_4$)	3.1 $\times$ 10
Ca^{++} ($CaCl_2$)	2.5 $\times$ 10^3
Mg^{++} ($MgSO_4$)	1.5 $\times$ 10^4
Na^+ (NaCl)	2.5 $\times$ 10^4

[a] Three injection series: 80-μg dose of cation in 0.2 ml of saline 24 hr prior to infection, on day of infection, and 24 hr after infection.

[b] LD_{50} values calculated by the method of Reed and Muench (21).

TABLE 2. *Effect of immunization on iron-enhanced infection with Listeria monocytogenes A4413*

Treatment[a]	LD_{50} value[b]
None (control)	2.5 $\times$ 10^3
Fe^{++}-treated	3.5 $\times$ 10
Immunized	1.8 $\times$ 10^6
Fe^{++}-treated immunized	4.9 $\times$ 10^6

[a] Three injection series: 80-μg dose of cation in 0.2 ml of saline 24 hr prior to infection, on day of infection, and 24 hr after infection.

[b] LD_{50} values calculated by the method of Reed and Muench (21).

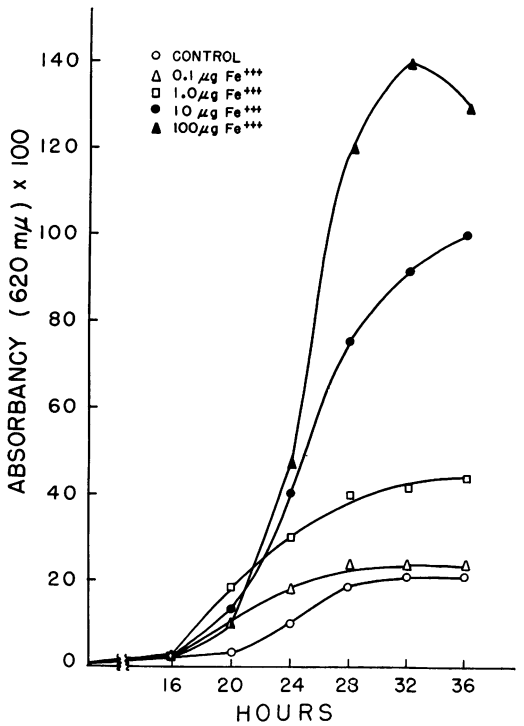

FIG. 1. *Effect of various concentrations of Fe^{+++} on in vitro growth curves of Listeria monocytogenes A4413.*

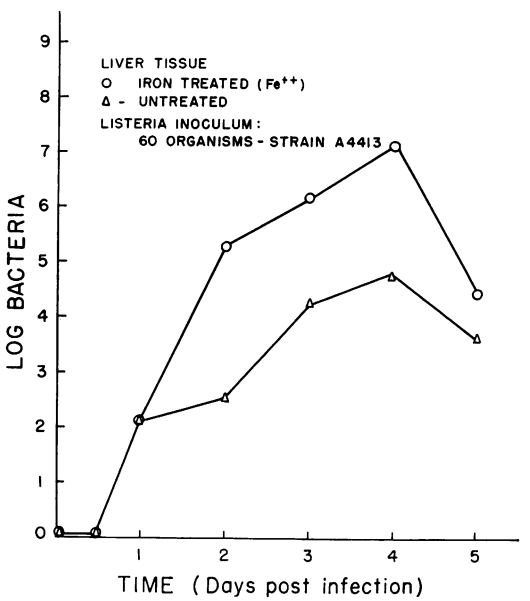

FIG. 2. *Growth of Listeria monocytogenes A4413 in liver of iron-treated and nontreated mice.*

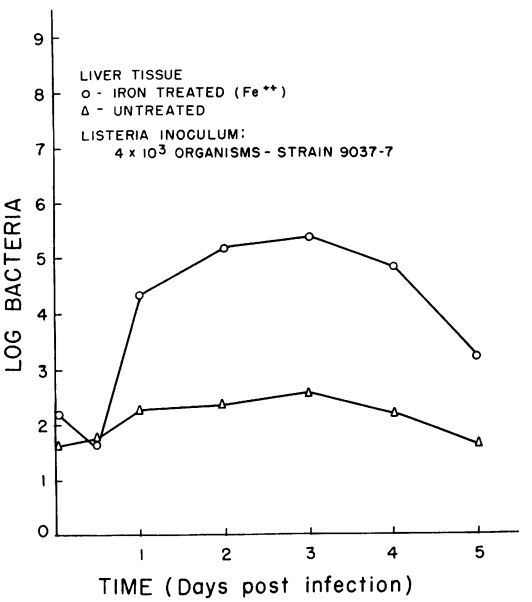

FIG. 3. *Growth of Listeria monocytogenes 9037-7 in liver of iron-treated and nontreated mice.*

ment with the avirulent strain 9037-7. This strain was not capable of sustained growth in either liver or spleen of nontreated mice, but multiplied in both tissues in iron-treated animals. Although strain 9037-7 multiplied and caused deaths in iron-treated mice, it did not reach the same numbers per gram of tissue as the virulent strain.

Circulatory and peritoneal clearance experiments. Bacterial clearance experiments were performed to determine whether the increased in vivo growth and reduction in LD_{50} dose were the result of damage to the RES in iron-treated mice. No differences in either circulatory or peritoneal clearance of *Listeria* cells were noted between iron-treated and nontreated animals.

Hemolytic anemia. Hemolytic anemia was induced to increase the availability of iron to the bacteria during infection. Within 24 hr after injection of 2.5 mg of phenylhydrazide or 16 HU of antimouse erythrocyte serum, the hematocrit values were reduced. Injection of saline or comparable dilutions of normal rabbit serum did not appreciably alter the hematocrit values of control animals. Varying dilutions of *Listeria* were injected into groups of animals with hemolytic anemia induced by injection of phenylhydrazine or antimouse erythrocyte serum. Mice with anemia induced by either method reacted with increased susceptibility to infection (Table 3).

Effect of Desferal treatment on infection. Desferal was used to deplete iron before and during infection. Desferal treatment appeared to have a

TABLE 3. *LD_{50} values of Listeria monocytogenes A4413 for mice with hemolytic anemia*

Group	Hematocrit	LD_{50} value[a]
	%	
Control (not treated)	44.6	3.1×10^4
Phenylhydrazine (2.5 mg)	28.8	3.9×10
Saline	47.6	8.5×10^4
Antimouse erythrocyte serum (16 HU)	38.9	0.46×10
Normal rabbit serum[b]	46.3	1.2×10^4

[a] LD_{50} values calculated by the method of Reed and Muench (21).

[b] Dilution comparable to that used for antimouse erythrocyte serum.

protective effect, as shown by an increase in LD_{50} dose (Table 4). Iron treatment of mice receiving Desferal reversed this effect and reduced the LD_{50} dose to comparable levels with iron-treated mice not receiving Desferal.

Plasma and tissue iron during infection and iron treatment. Plasma iron decreased during progressive infection (Table 5). Table 6 shows the levels of iron in the liver and spleen. Although the mean hepatic iron values increased during infection, the wide range and large standard errors made it difficult to establish with certainty the extent of any changes. Splenic iron remained essentially the same during infection.

Iron treatment of mice increased the mean hepatic and splenic iron values, but did not affect plasma iron. A wide range of tissue iron values, comparable to those during infection, was observed.

DISCUSSION

Treatment of mice with Fe^{+++} or Fe^{++} consistently reduced the LD_{50} values for *L. monocytogenes*. This effect was produced by three ip injections in saline rather than a single injection of the salts in oil, as had been done with *P. pestis* by Jackson and Burrows (11). It was feared that use of oil or other such material might induce an inflammatory exudate. Oral administration of iron compounds was attempted in preliminary experiments, but the normal mechanisms controlling iron absorption by the intestinal mucosa did not make this method feasible. Dietary iron is usually absorbed only if the body has a specific need for it; otherwise, it is excreted (5, 7). Massive doses of iron by the oral route would cause some increased absorption, but would be difficult to control. Other cations such as Ca^{++}, Mg^{++}, and Na^{+} did not affect the LD_{50} dose, or raised it slightly. Mn^{++} caused a reduction in LD_{50} dose much like that of iron, but was inhibitory to growth of *Listeria* in vitro. The concentration of Mn^{++} in tissue is normally not of the same magnitude as iron. The human body contains 160 μg of Mn^{++} per kg of lean tissue (6), whereas it contains 50 to 60 mg of iron per kg (8). Manganese has been shown to be transported in the body by a β globulin, possibly

TABLE 4. *Effect of iron chelation with Desferal on the LD_{50} of Listeria monocytogenes A4413*

Treatment	LD_{50} value[a]
None (control)	3.7×10^4
Fe^{++}[b]	0.8×10
Fe^{++}[b] and Desferal	1.3×10^2
Desferal	1.1×10^6

[a] LD_{50} values calculated by the method of Reed and Muench (21).

[b] Three injection series: 80-μg dose of Fe^{++} in 0.2 ml of saline 24 hr prior to infection, on day of infection, and 24 hr after infection.

TABLE 5. *Plasma iron levels during infection with Listeria monocytogenes A4413*

Hr after infection[a]	Plasma iron[b] (μg/100 ml)
0	237 ± 6 (3)
24	163 ± 9 (5)
48	162 ± 4 (5)
72	130 ± 4 (3)
Control (not infected)	233 ± 8 (5)

[a] The infecting dose consisted of 10^5 organisms.

[b] Mean ± SE; parentheses indicate number of determinations each performed on pooled plasma from 10 to 15 mice.

TABLE 6. *Iron levels in mice during infection with Listeria monocytogenes A4413*

Hr after infection[a]	Liver		Spleen	
	No. of animals	Iron[b] (μg of Fe/g)	No. of animals	Iron[b] (μg of Fe/g)
0	13	85 ± 8.3 (45–130)	11	101 ± 6.6 (74–145)
24	11	80 ± 10.0 (30–120)	12	98 ± 7.2 (66–137)
48	12	101 ± 7.8 (50–135)	13	99 ± 8.0 (58–145)
72	12	108 ± 7.8 (82–162)	12	91 ± 9.3 (60–158)

[a] Infecting dose consisted of 10^5 organisms.

[b] Mean ± SE; the range of values is given in parentheses.

transferrin (6), and, therefore, may have been interfering with iron metabolism in a manner favorable to the bacteria, perhaps by replacing iron in transferrin.

Iron has been shown to exert a stimulatory effect on in vitro growth of *Listeria*. The site of incorporation or utilization within the bacterial cell was not determined. Keeler and Gray (14) showed incorporation of Fe^{59} into cell wall fractions contaminated with cell membrane and into the protoplasmic fraction of *L. monocytogenes*. It is probable that iron is being incorporated into the cytochromes usually associated with the bacterial cell membrane and into catalase. In experiments to be reported in a later paper, a major portion of Fe^{59} from the medium was incorporated into the cells during the initial stages of growth and reappeared in the medium during the logarithmic phase of growth (Jackson and Sword, *unpublished data*).

L. monocytogenes grew more rapidly and to a greater extent in iron-treated than in nontreated mice. This is suggestive evidence that death in these animals was through mechanisms associated with the increased bacterial population rather than production of factors having pronounced toxic activity. If iron induced or was needed for incorporation into a toxic factor, it would be expected that deaths would have occurred much earlier and with considerably lower bacterial counts than in the nontreated groups. This hypothesis is consistent with the increased virulence of the supposedly avirulent strain, 9037-7, in iron-treated mice, and stimulation of growth in iron-supplemented media (Fig. 2).

L. monocytogenes is a facultative intracellular parasite, and immunity to listeriosis appears to be due, totally or in great part, to cellular rather than humoral factors (1, 15). Iron-treated mice had a level of resistance to challenge comparable to nontreated immunized animals. Phagocytic cells are known to remove iron from plasma and to serve as storage depots for iron in the form of ferritin (28). *Listeria* cells have been observed to be surrounded by ferritin within phagosomes of mouse spleen macrophages (2). If we assume that iron exerts a stimulatory effect during intracellular growth of *Listeria*, and that "immune" phagocytes destroy the organism rapidly, as shown by Mackaness (15), then the organisms would perhaps not have time to utilize iron stores of the "immune" cell before they are destroyed.

Phenylhydrazine and antimouse erythrocyte serum increased susceptibility to listeriosis. The results are similar to those reported by Kaye and Hook (13) with *Salmonella typhimurium*. However, these authors suggest that phagocytosis of altered erythrocytes by reticuloendothelial cells impairs their capacity to kill ingested bacteria. It had been shown previously by others that injection of particulate material may cause stimulation of phagocytic activity by the RES and that pretreatment of rats with albumin-globulin aggregates, produced by heating, causes a stimulation of the RES capable of protecting animals against experimental infection with *S. typhimurium* (4). Hemolytic anemia caused by phenylhydrazine intoxication is known to cause a rise in serum iron levels within 3 to 5 hr after injection (8). It is also established that inflammation and infection may cause fixation and accumulation of iron in the cells of the RES (8, 28). Thus, increased availability of iron rather than decreased bacterial destruction resulting from damage to the RES may be responsible for the increased susceptibility in animals with hemolytic anemia.

Desferal has been shown to remove iron from transferrin and to deplete iron stores in tissue (18). This compound appeared to make mice more resistant to *Listeria* infection, perhaps by limiting the growth of the organisms and by allowing the host defenses time to overcome the bacteria.

The decrease observed in plasma iron during progressive infection may be a reflection of iron fixation in tissue. The mean values obtained for hepatic iron are suggestive of hepatic iron accumulation during infection. The wide range of values for tissue iron was confirmed in other experiments and is consistent with iron values reported by others in rabbit liver (26).

L. monocytogenes produces a soluble hemolysin with properties similar to streptolysin O (10, 12, 20). The hemolysin is reported to be nontoxic for intact animals, lytic for leukocytes, and to produce opacity in egg-yolk medium, lecithovitellin, or serum. Destruction, in vivo, of erythrocytes or leukocytes could increase the amount of iron available to the bacteria, and thereby enhance the infection. The effect of purified hemolysin on levels of serum iron, hemoglobin, transferrin, and haptoglobin has not yet been investigated.

Endocrine disorders can have a pronounced effect on iron metabolism and erythropoiesis (8). Direct and indirect stimulation of the adrenal cortex is reported to cause elevated serum iron values. Cortisone was reported by Miller and Hedberg (17) to cause a reduction in LD_{50} of *L. monocytogenes* from 10^4 or 10^5 to less than 50 bacteria. Although cortisone may also cause other effects which would lower resistance of the animal, its effect on iron metabolism is consistent with involvement of iron in the pathogenesis of listeriosis. If availability of iron in host tissue is involved in pathogenesis of naturally occurring listeriosis, then stress and other factors which affect endocrine balance may influence suscep-

tibility to listeriosis through induction of changes in iron metabolism.

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