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Neisseria meningitidis Infection in Mice: Influence of Iron, Variations in Virulence Among Strains, and Pathology

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The influence of iron on *Neisseria meningitidis* infection in C-57 mice was examined. Iron sulfate, ferric ammonium citrate, and iron sorbitol citrate all proved to be too toxic for use as infection-enhancing agents. Iron dextran displayed an extremely low toxicity, enhanced infection in a dose-dependent manner, and resulted in infection enhancement factors approaching 10^9 for virulent strains of *N. meningitidis*. Fatal iron dextran-enhanced infection was shown to be reversible by in vivo chelation of iron. Virulent strains of *N. meningitidis* produced symptoms of infection and pathological lesions in mice both with and without iron pretreatment, but an avirulent strain failed to produce symptoms of infection or pathological lesions, regardless of iron administration. Iron dextran-enhanced infection in mice proved to be a useful model for the examination of virulence of various *N. meningitidis* strains. All of 9 isolates from clinical disease possessed virulence, whereas only 3 of 10 isolates from carriers possessed virulence, when examined by using this model.

Neisseria meningitidis infection in mice, enhanced by mucin (8), has been shown to be useful in the study of relative virulences of *N. meningitidis* strains (9). However, mucin is chemically ill defined, and preparations differ in their infection-enhancing capabilities (2, 6).The chelation of iron from mucin has been shown to reduce the infection-promoting activity of mucin, and various other forms of iron have promoted fatal infection from *N. meningitidis* in mice (2). However, the dosages of iron sulfate or iron sorbitol citrate required to effect a significant enhancement of infection approached toxic levels for these iron compounds. Thus, iron toxicity may have been responsible for some of the observed mortality.

Considerable evidence does exist that iron plays an important role in host-parasite relationships, involving a number of bacterial pathogens (11, 14). The pathogenesis of meningococcal infection in humans and laboratory animals is poorly understood. Therefore, it was considered worthwhile to evaluate the influence of iron on meningococcal infection in mice.

MATERIALS AND METHODS

Bacterial strains. The 20 strains of *N. meningitidis* used during the course of this study are listed in Table 1, with their serogroups and sources. Both disease strains (i.e., strains obtained from cases of overt meningitis) and carrier strains (i.e., strains obtained by nasopharyngeal swabbing of asymptomatic carriers) were included. Eleven of the Defence Research Establishment Suffield (DRES) strains were isolated in 1974 at Canadian Forces Recruit School Cornwallis during a period marked by three cases of overt meningitis. The other two DRES strains were obtained locally. Two strains, SP3424 and SP3428, were originally isolated from disease cases in São Paulo, Brazil, and were obtained from I. W. DeVoe (4). The Laboratory Centre for Disease Control (LCDC) strains represent disease isolates from various parts of Canada, and these were obtained, along with the Slaterus strain, from F. E. Ashton (LCDC, Ottawa, Canada). DRES-17 was isolated from the air of a recruit barracks by using a large-volume air sampler (15). All strains were lyophilized. Cultures were grown on Columbia blood agar (CBA) (Columbia blood agar base [Grand Island Biological Co., Grand Island, N.Y.], containing 4% [vol/vol] sheep erythrocytes and IsoVitaleX [Baltimore Biological Laboratory, Cockeysville, Md.]), in tissue culture flasks (75-cm^2 growth area, Falcon, Oxnard, Calif.) at 35°C and in an atmosphere containing 5% (vol/vol) CO_2 in air for 16 to 18 h. Growth was dislodged from the agar surface by gentle rocking in 5 ml of *Neisseria* chemically defined medium (NCDM) (Grand Island Biological Co.) containing approximately 20 (5-mm-diameter) glass beads. Harvested cell suspensions contained approximately 10^{10} colony-forming units (CFU) of *N. meningitidis*. Dilutions of cell suspensions were prepared in NCDM. Viable counts were determined as CFUs, after 18 to 24 h of growth on CBA at 35°C in 5% CO_2-air. Heat-killed cells were prepared by heating cell suspensions at 70°C for 15 min.

TABLE 1. *Virulence of N. meningitidis strains*[a]

Strain	Serogroup	Source	LD_{50}[b] (CFU)	Virulence
SP 3428	A	Disease[c]	9.1×10^4	High
SP 3424	A	Disease[c]	2.3×10^2	High
604	A	Disease[d]	2.0×10^6	Low
608	B	Disease[d]	1.0×10^3	High
DRES-01	B	Disease[e]	6.9×10^1	High
13090	B	Disease[f]	1.1×10^6	Low
DRES-02	C	Disease[e]	6.2×10^6	Low
DRES-06	C	Disease[e]	2.5×10^6	Low
2241	C	Disease[d]	1.0×10^0	High
DRES-03	B	Carrier[e]	$>10^8$	None
DRES-07	B	Carrier[e]	4.4×10^4	High
DRES-18	B	Carrier[e]	$>10^8$	None
DRES-04	C	Carrier[e]	$>10^8$	None
DRES-05	C	Carrier[e]	$>10^8$	None
DRES-14	C	Carrier[e]	3.6×10^1	High
DRES-08	Y	Carrier[e]	$>10^8$	None
DRES-15	Y	Carrier[g]	2.6×10^5	Low
Slaterus	Y	Carrier[d]	$>10^8$	None
DRES-19	W	Carrier[g]	$>10^8$	None
DRES-17	B	Aerosol[e]	1.0×10^2	High

[a] Mice in groups of 5 to 10 were pretreated with iron dextran (250 mg of Fe per kg).

[b] LD_{50}'s determined by probit analysis of 72-h mortality data.

[c] São Paulo, Brazil.

[d] LCDC.

[e] Cornwallis, Canada.

[f] ATCC.

[g] Local.

Mice. C-57 black male mice (HPB strain [2]) were obtained from the University of Calgary, Canada. Mice of 18 to 22 g and of the same age were placed in groups of 5 to 20 animals. Mice of any group weighed within 2 g. Mice were fed (Lab-Blox, Allied Mills Inc., Chicago, Ill.) and watered ad libitum throughout all experiments.

Iron compounds. The following iron compounds were tested for their usefulness as infection enhancers: ferrous sulfate (Fisher); ferric ammonium citrate (British Drug Houses Ltd.); iron sorbitol citrate (Jectofer, Astra Pharmaceuticals, Mississauga, Canada), and iron dextran (Dextran Products Ltd., Scarborough, Canada). The compounds were diluted in physiological saline. Iron concentration was quantified with a Jarrell-Ash atomic absorption spectrometer (flame absorption mode). Toxicities of iron compounds were measured as 50% lethal doses (LD_{50}'s) in terms of Fe at 48 h after intraperitoneal (i.p.) injection.

Infection studies. Groups of mice were pretreated by injecting i.p. 0.5 ml of either iron sorbitol citrate or iron dextran, and this was followed immediately by i.p. injection of 0.5 ml of a bacterial suspension. Infecting doses ranged from 1 to 10^9 CFU per mouse. Controls received Fe only or the highest dose of bacteria only. Additional controls for some experiments included groups receiving the equivalent of 10^9 CFU of heat-killed cells of strain 2241, with pretreatment of 250 mg of Fe per kg as iron dextran, and groups pretreated with 310 mg of dextran per kg (Fe-free dextran, a dosage equivalent to that from 250 mg of Fe per kg of iron dextran, Dextran Products Ltd.) before being infected with strain 2241. Experiments lasted for 72 h, at which time cumulative mortality data were determined for probit analysis by computer to derive the LD_{50} in terms of CFU for each experiment.

Mice of some experiments involving strain DRES-01 were studied in more detail. Clinical observations were made at 3- or 6-h intervals during the first 24 h. Rectal temperatures were also measured on at least 10 mice per group with an Ellab electronic thermometer. Two mice per group were bled by heart puncture with 2-ml Vacutainers (Becton Dickinson), 0.1-ml samples of the blood were diluted in NCDM, and the CFU per milliliter of blood was determined. The spleens of the bled mice were weighed and homogenized in 3 ml of NCDM for 1 min at 37,000 rpm in a Sorval omnimixer (microattachment). Spleen homogenates were diluted in NCDM, and the CFU per gram of spleen was determined.

Once investigations of iron compounds had been completed, a standard Fe dosage was administered for comparing virulence of strains. Thereafter, animals were always pretreated with iron dextran, at a dosage of 250 mg of Fe per kg, immediately before challenge with bacteria. Controls received the highest dose of bacteria but no iron. Strains were arbitrarily considered to be avirulent if their LD_{50}'s were in excess of 10^8 CFU, to possess low virulence if their LD_{50}'s fell in the range of $>10^5$ to $<10^8$ CFU, and to possess high virulence if their LD_{50}'s were $<10^5$ CFU.

Reversal of iron-enhanced infection by deferoxamine mesylate. Mice were pretreated with iron dextran at 250 mg of Fe per kg and infected with various levels of CFU of strain 2241. At intervals of 0 to 6 h, 25 mg (1,250 mg/kg) of deferoxamine mesylate (Desferal, Ciba-Geigy, Dorval, Canada) in physiological saline was injected i.p. (The toxicity of deferoxamine mesylate by the i.p. route was >1,600 mg/kg; Holbein et al., unpublished data.)

Pathology. Mice for histopathological study had received iron dextran over the range of 15 to 250 mg of Fe per kg and had been infected with varying numbers of CFUs of the following strains: DRES-01, DRES-02, DRES-06, 604, 608, 2241, and Slaterus. Mice receiving either cells or iron dextran were also included. Mice were sacrificed at various stages of terminal illness and after apparent recovery from infection, usually at 48 to 72 h postinjection. In addition, mice receiving 250 mg of Fe per kg as iron dextran were examined over a period of 1 month. A total of 52 animals from the various experiments were necropsied. Changes in shape, size, and color of kidney, spleen, liver, lung, and brain were noted. The tissues of 52 livers, 52 spleens, 23 lungs, 35 kidneys, and 23 brains were submerged in phosphate-buffered saline containing 3.7% (vol/vol) Formalin. Paraffin sections, 5 μm thick, were cut and stained with hematoxylin-eosin.

RESULTS

Influence of iron on infection. It was con-

sidered essential to first examine the toxicities of the various iron compounds before examining their effects on *N. meningitidis* infection in mice so that an iron compound of low toxicity might be selected. Iron sulfate, ferric ammonium citrate, and iron sorbitol citrate all possessed high degrees of toxicity for the mice (Table 2). Ferric ammonium citrate was considered to be too toxic to warrant further investigation. Iron sorbitol citrate appeared to enhance infection at a level of 15 mg of Fe per kg with strain DRES-01. The LD_{50} for strain DRES-01 was lowered from $>10^8$ to 8×10^2 CFU under these conditions, but controls receiving 15 mg of Fe per kg only were observed to suffer a 10 to 20% mortality rate. At a reduced level of 7.5 mg of Fe per kg, iron sorbitol citrate failed to significantly enhance infection ($LD_{50} > 10^6$ CFU). Both iron sorbitol citrate and ferrous sulfate were abandoned as infection-enhancing agents due to their toxicities (Table 2).

Iron dextran was found to exhibit a very low toxicity for mice (Table 2), and it was without apparent ill effect at a dosage of 1,600 mg of Fe per kg. Iron dextran enhanced infection with strain DRES-01 in a dose-dependent manner (Fig. 1). A level of 250 mg of Fe per kg provided a high degree of enhancement, with mice succumbing to fatal infection when as little as 10 CFU of strain DRES-01 was injected. These results, together with the fact that the required level of iron was far below toxic levels, prompted us to further examine iron dextran-enhanced infection.

Iron dextran-enhanced infection. Infection in mice pretreated with 250 mg of Fe per kg as iron dextran and infected with approximately 10^3 CFU of strain DRES-01, could be detected as bacteremia as early as 3 h postinjection (Fig. 2). Bacterial counts in the blood reached approximately 10^7 CFU per ml within 6 h postinjection and remained at this high level at least until 24 h postinjection. Bacterial counts in the spleen also followed this pattern. Symptoms of infection could be readily observed at 6 h post-

TABLE 2. *Toxicities of iron compounds*

Compound	No. of determinations	LD_{50}[a] (mg of Fe per kg)
Ferrous sulfate	2	25
Ferric ammonium citrate	1	<12.5[b]
Iron sorbitol citrate	3	22.5
Iron dextran	2	>1,600[c]

[a] Mortality scored at 48 h after i.p. injections of iron compounds in saline, groups of 5 to 10 mice.
[b] Lowest dose examined, complete kill.
[c] Highest dose examined, no kill.

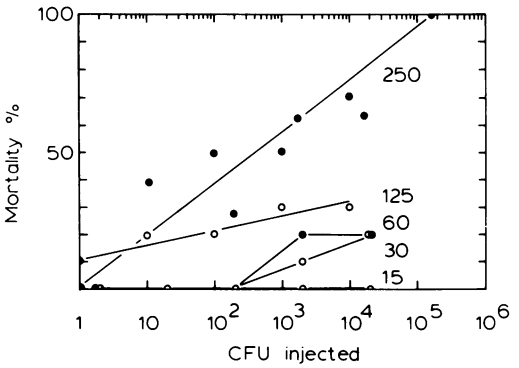

FIG. 1. *Enhancement of N. meningitidis infection by iron dextran. Mice in groups of 10 to 20 animals were pretreated with iron dextran at the dosages indicated (milligrams of Fe per kg) and infected with strain DRES-01. Mortalities were scored at 72 h post injection.*

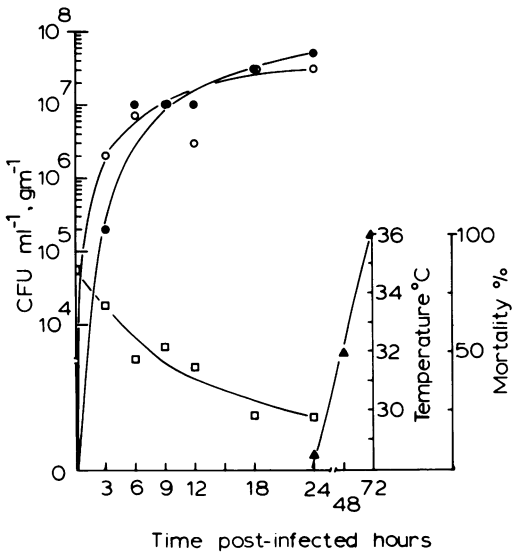

FIG. 2. *Course of iron dextran-enhanced infection. Mice were pretreated with 250 mg of Fe per kg and infected with 10^3 CFU of strain DRES-01 at zero time. During infection, bacteria levels in blood (●) and spleen (○) were determined. Rectal temperatures (□) were also taken, and mortality (▲) was scored.*

injection. These symptoms included: lachrymation, inactivity, and hypothermia (Fig. 2). Within 12 h the eyes were covered with a white mucous-like discharge, hair was "brittle" and rough, and a clear mucous-like discharge was evident from the anus. The bulk of the animals died between 24 and 72 h post injection (Fig. 2). Not all clinically affected mice would die. Survivors at 72 h did not succumb during a further 7 days.

Control animals injected with $>10^7$ CFU of

strains 2241 or DRES-01 but no iron had similar but less severe symptoms of infection and fatal infection in these mice did not occur until approximately 10^8 to 10^9 CFU had been injected. Heat-killed cells of strain 2241 were incapable of causing death in iron-pretreated animals at a dose equivalent to 10^9 CFU. The iron-free dextran used to manufacture iron dextran was incapable of enhancing infection with strain 2241 ($LD_{50} > 10^8$ CFU). Strain 2241 caused fatal infection in the absence of iron pretreatment when more than 10^8 CFU was injected. The LD_{50} for strain 2241 was lowered from approximately 5×10^8 to 1 CFU in the presence of iron, an enhancement factor approaching 10^9.

Deferoxamine mesylate, an iron chelator used clinically to treat iron intoxication, prevented fatal iron-enhanced infection with strain 2241 (Table 3). The chelator was most effective when given soon after the mice had been infected and only when 10^3 CFU or less had been injected. The amount of chelator administered theoretically chelated 40% of the injected iron. Similar results were also obtained with strain DRES-01 (data not shown).

Virulence of strains. The virulences of 20 strains of *N. meningitidis* were examined to assess the usefulness of iron dextran-enhanced infection as a model system for strain comparisons. All nine disease strains examined proved to possess virulence (Table 1), and strains of both high and low virulence were evident from serogroups A, B, and C. The most virulent strains examined were SP 3424, DRES-01, and 2241 (representing serogroups A, B, and C, respectively). Only 3 out of 10 carrier strains had detectable virulence (Table 1). Virulence was detectable in carrier strains of serogroups B, C, and Y. Strain DRES-17, which had been collected from the air of a recruit barracks during a period of overt disease, also had high virulence. LD_{50}'s in the absence of iron were greater than 10^8 CFU for all of the strains shown in Table 1.

TABLE 3. *Effects of deferoxamine mesylate on iron-enhanced infection*

CFU injected[a]	% Mortality[b] at time of deferoxamine mesylate administration (h postinfection)					
	0	1	2	3	6	Control
1.0×10^1	10	0	—[c]	—	0	40
1.0×10^3	40	30	—	30	80	100
2.5×10^5	45	100	100	100	90	100

[a] Strain 2241.

[b] Mice in groups of 10 to 20 animals were pretreated with 250 mg of Fe per kg (iron-dextran), infected at zero time, and given 25 mg of deferoxamine mesylate at the times indicated. Controls received no deferoxamine mesylate. Mortalities were scored at 72 h postinfection. (Average results from two experiments.)

[c] —, Not determined.

Pathology of infection. Significant pathological changes were not observed in any control mice receiving iron dextran up to 250 mg of Fe per kg. Pathological changes were not observed in the brains, lungs, or kidneys of any infected mice and in any of the tissues of mice infected with the avirulent strain Slaterus. Pathological changes were readily observed in the livers and spleens of mice infected with virulent strains of *N. meningitidis*, both in the absence and in the presence of iron dextran. Spleen changes were detected macroscopically as dark lesions (13%) and enlargement (40%). Liver changes were detected as a mottled appearance with cream-colored, raised lesions (45%). Twenty-eight percent of the spleens had thrombi in veins and/or necrosis in germinal centers and general depletion of lymphoid tissue (Fig. 3A). Twenty-seven percent of the livers had thrombi in veins (Fig. 3B) and/or caseous necrosis of associated hepatic tissue with peripheral infiltration of mononuclear cells. Focal areas of hepatic necrosis, with infiltration of mononuclear cells, were also evident (Fig. 3B). These latter lesions appeared not to be directly associated with thrombi.

DISCUSSION

Iron sulfate and iron sorbitol citrate have been previously shown to provide marked enhancement of fatal *N. meningitidis* infection in mice when given at dosages of approximately 25 and 15 mg of Fe per kg, respectively (2). The results of the present study confirm that a dosage of 15 mg of Fe per kg as iron sorbitol citrate is necessary to achieve significant enhancement of infection. However, the present results also indicate that this is near a toxic dose for the iron alone, for both iron sorbitol citrate and iron sulfate. Thus, the near toxic levels of iron used by Calver et al. (2) may explain their failure to detect pathological evidence for infection, other than bacteremia. Ferric ammonium citrate was also found to be highly toxic, confirming the results of Ford and Hayhoe (6). The influence of ferric ammonium citrate on *N. meningitidis* infection was not examined, although at levels below a toxic dose it has been shown to enhance *Vibrio cholerae* (6) and *Salmonella typhimurium* (7) infection in mice. The use of iron sulfate, iron sorbitol citrate, and ferric ammonium citrate was abandoned for the purposes of the present study to avoid any complications from toxicity.

Iron dextran was found to possess an extremely low toxicity for mice (LD_{50} greater than

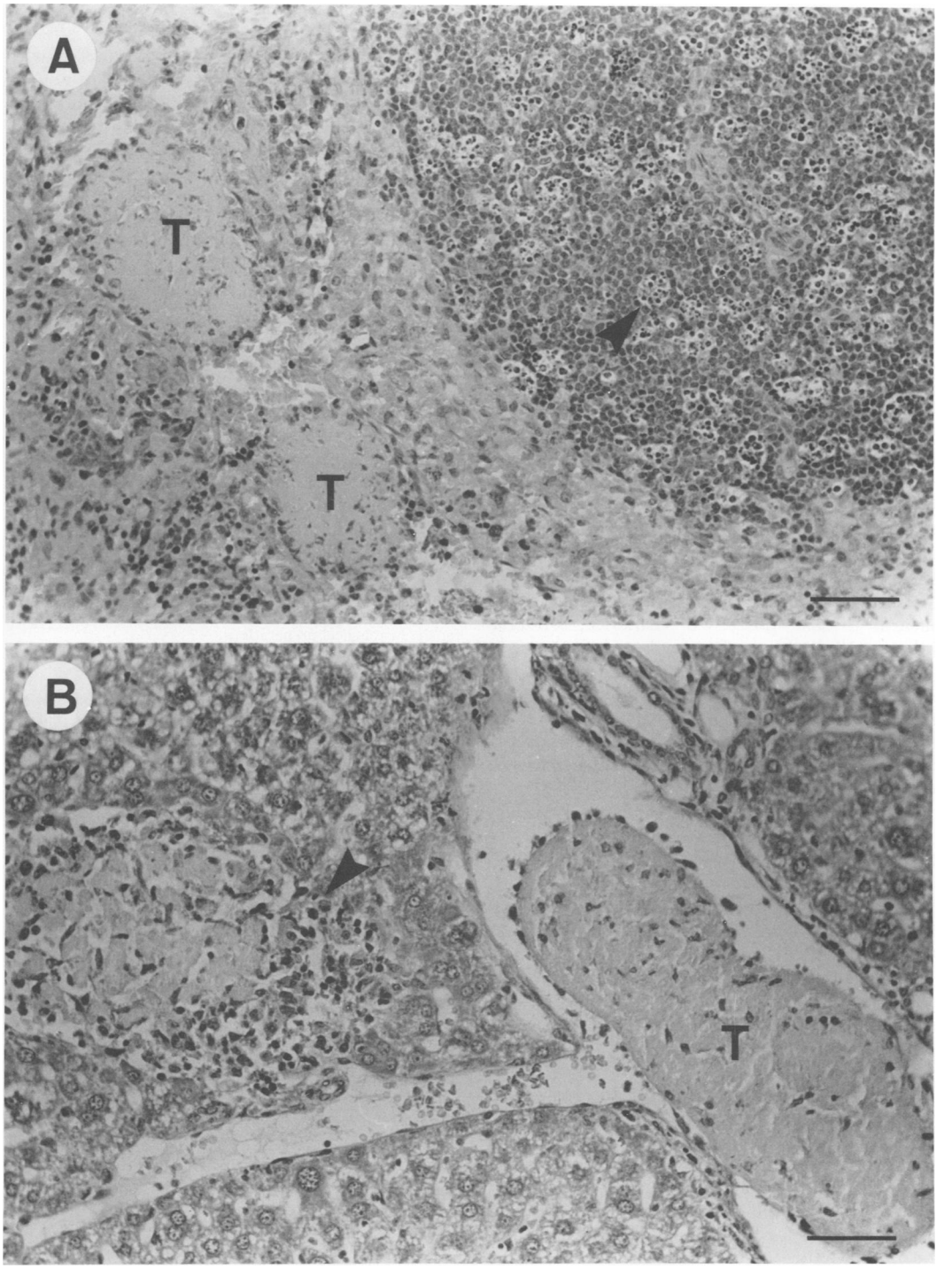

FIG. 3. *Pathological features of N. meningitidis infection in mice. (A) Spleen taken from a mouse 48 h after pretreatment with 250 mg of Fe per kg (iron dextran) and infection with 4.5 × 10^1 CFU of strain DRES-06. Thrombi (T) are evident as well as necrosis of lymphoid follicles (arrow). Bar = 50 μm. (B) Liver taken from a mouse 48 h after infection with 2.6 × 10^5 CFU of DRES-01. No iron pretreatment had been used. A thrombus (T) undergoing organization is visible as well as an area of necrosis of hepatic parenchyma surrounded by mononuclear cells (arrow). Bar = 50 μm.*

2,000 mg of Fe per kg [1]) and to enhance fatal *N. meningitidis* infection in mice in a dose-dependent manner. The virulence of strain 2241 was enhanced by a factor approaching 10^9 when mice were pretreated with iron dextran at 250 mg of Fe per kg. The requirement for the relatively high dose of iron remains obscure, but it seems undoubtedly related to its composition. The iron of iron dextran has been shown to be less mobile than that of iron sorbitol citrate (13) and enzymes linked to dextran have prolonged half-lives in the body (12). This evidence suggests that iron dextran does not enter the physiological iron pools as quickly as other forms, and this contention is supported by the very low toxicity of iron dextran. Payne and Finkelstein (11) also used a comparatively high dose of iron dextran as an infection-enhancing agent in chicken embryos. That iron dextran enhanced infection at dosages far below a toxic dose makes it an attractive agent for infection enhancement.

Overt symptoms of infection in iron-treated mice were only observed with virulent strains of *N. meningitidis*. In addition, all 9 strains from natural disease possessed virulence, whereas only 3 of 10 carrier strains displayed virulence in this model. The symptoms of infection in the mice were suggestive of endotoxemia. It is interesting to note in this regard that Moore et al. (10) have also observed a progressive hypothermia attributable to endotoxin in mice infected with *S. typhimurium*.

The principal pathological evidence for infection included thrombosis of veins in both the liver and spleen, with necrosis of associated tissue, and lymphoid degeneration in the spleen. Intravascular coagulation (thrombosis) has been demonstrated in rabbits treated with *N. meningitidis* endotoxin (3), and this phenomenon is observed in fulminating human disease (5). The avirulent strain Slaterus failed to produce pathological changes, but all the virulent strains examined produced pathology indicative of endotoxin action.

Several pieces of evidence from this study suggest that iron from iron dextran enhances *N. meningitidis* infection in mice. Heat-killed cells of a virulent strain, at a dose equivalent to 10^9 CFU, in combination with iron, were without effect, indicating that preformed endotoxin was not responsible for fatality. The dextran used in the manufacture of iron dextran also failed to enhance infection. The participation of iron in the infectious process was demonstrated by the use of deferoxamine mesylate, which blocked fatal infection even when administered at 3 h postinfection, when bacteremia was evident. The finding of pathological evidence of infection in the livers and spleens of mice infected with virulent strains, but receiving reduced or no iron pretreatment and displaying little or no symptoms of infection, is highly significant. This evidence suggests that exogenous iron may not be mandatory for the infectious process, but rather may modulate the severity of infection by supplying iron to bacteria and/or by increasing the susceptibility of mice to infection. Thus, nonfatal infection may occur with virulent strains at levels of exogenous iron far below that required to produce a fatal infection. Iron may also serve to render the host more susceptible to infection by impairing defense mechanisms (e.g., interference with the reticuloendothelial system). These possibilities are presently being investigated.

N. meningitidis infection in mice, enhanced by iron dextran, may prove to be a useful model in which to study both the role of iron in infection and the physiological bases of virulence of *N. meningitidis*.

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