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Enhancement of *Neisseria meningitidis* Infection in Mice by Addition of Iron Bound to Transferrin

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Small quantities of iron bound specifically to human transferrin were found to stimulate infection with *Neisseria meningitidis* strain M1011 in mice. An intraperitoneal injection of 17.5 mg of transferrin carrying 22.7 μg of Fe resulted in 100% mortality from infection, as compared with no mortality for the controls which had received saline. Five milligrams of ferri-transferrin (FeTf), carrying 6.5 μg of Fe, stimulated and prolonged bacteremia in the mice. Thus, FeTf maintained infection, whereas infection was controlled due to iron limitation in control mice. Comparative studies with apotransferrin (iron-free) revealed that the enhancement of infection was due to the supply of iron. FeTf was also found to relieve an iron limitation of growth achieved by ethylenediaminedihydroxyphenylacetic acid (EDDA) in vitro. FeTf abolished the lag phase for growth of *N. meningitidis* in a defined medium. The results of this study suggest that human FeTf is an immediate source of iron to *N. meningitidis* both in vitro and in vivo. These findings support the hypothesis that the levels of iron in the circulating transferrin pool of mice determine the course of experimental *N. meningitidis* infection.

Iron availability is a major determinant of susceptibility to bacterial infection because bacteria have absolute iron requirements and iron is relatively unavailable within vertebrate hosts (8, 14, 15). Body iron stores are associated with two major classes of proteins which display high affinities for iron: the transferrins and the ferritins (2).

Serum transferrin is a glycoprotein and plays a critical role in the transport of iron among its sites of absorption, storage, and utilization, whereas ferritins are found primarily in tissues and are thought to function mainly in iron storage (2). Iron of the circulating transferrin pool could be extremely important during infection due to its pivotal role.

Serum contains approximately 3 mg of transferrin per ml, which can bind a total of approximately 4.2 μg of Fe and therefore could represent an important source of iron to invading bacteria. However, serum transferrin is usually only about 25% saturated with iron and has an association constant for iron of approximately 10^{36}. This results in a net free serum iron concentration of about 10^{-18} M (8), which is too low for bacterial growth (8, 15). Bacterial pathogens must utilize iron acquisition mechanisms capable of competing with transferrin to grow in normal serum. An enterochelin-producing strain of *Salmonella typhimurium* grew in human serum containing partially unsaturated transferrin, whereas an enterochelin-negative strain required exogenous iron for growth in the serum (16). Similar results have been reported for *Escherichia coli* (12). The preceding evidence suggests that siderophores, such as enterochelin, might facilitate iron acquisition from transferrin if elaborated in vivo. Added enterochelin did enhance the apparent virulence of *S. typhimurium* (16), and enterochelin has been recovered from guinea pigs infected with *E. coli* (9). *Neisseria meningitidis* has been shown to obtain iron directly from human transferrin in vitro, apparently without the involvement of a cell-free siderophore (4, 5).

Transferrin also appears to play a central role in the redistribution of iron which can occur during infection (7, 8, 15). Studies of bacterial infections in human volunteers showed that plasma iron levels dropped soon after the initiation of infection (6, 7). Beisel (6) has reviewed evidence suggesting that iron removed from the transferrin pool during infection is stored in the liver iron stores. This overall response of iron sequestration is thought to deprive invading microorganisms of essential iron (8, 15).

Previous studies at this laboratory demonstrated that added iron dextran enhanced *N. meningitidis* infection in mice (10, 11). The principal role for the added iron appeared to be the maintenance of plasma transferrin iron levels during infection (10). In addition, an iron-con-

trolled infection was obtained in untreated mice, where plasma transferrin iron disappeared in concert with infection (10). Thus, iron sequestration was occurring during infection of normal mice. These findings suggested a major role for transferrin iron as an iron source for *N. meningitidis* during infection.

In this study, the capacity of human transferrin to stimulate *N. meningitidis* infection in mice was examined. The results indicated that small amounts of Fe on transferrin stimulated bacteremia and produced fatal infection. Apotransferrin (ApoTf; iron-free) was without effect. Transferrin was also found to relieve ethylenediaminedihydroxyphenylacetic acid (EDDA)-mediated, iron-limited in vitro growth of *N. meningitidis*.

MATERIALS AND METHODS

Bacterial strain. *N. meningitidis* strain M1011, a serogroup B, serotype 2,10 disease isolate, was used. Maintenance and cultivation conditions were as described before (10, 11). This strain was highly virulent for mice given iron dextran, with a 50% lethal dose of <2 colony-forming units (CFU) (10). Cultures grown for 16 h on Columbia blood agar were harvested, diluted in neisseria chemically defined medium (NDM), and used to infect the mice as described before (10, 11).

Mice. C57 black male mice were maintained as described before (11), and all bacterial and transferrin injections were made intraperitoneally.

Preparation of ApoTf and FeTf. Human transferrin (Sigma Chemical Co., St. Louis, Mo.) was used for this study. All lots of the transferrin were found to yield single precipitin bands when examined by immunoelectrophoresis against rabbit anti-human serum (GIBCO Laboratories, Grand Island, N.Y.). ApoTf was prepared by dialysis against a citrate-acetate buffer, pH 4.5, as described by Aisen et al. (1) followed by 6 h of dialysis against 0.15 M NaCl, pH 7.0. ApoTf preparations were sterilized by passing them through filters (0.45-μm pore size; Millipore Ltd., Mississauga, Canada).

Ferri-transferrin (FeTf) was prepared in 40 mM tris (hydroxymethyl) aminomethane-hydrochloride buffer, pH 7.4, containing 2 mM $NaHCO_3$. The buffer contained 0.025 μmol of $FeCl_3$ complexed with 0.25 μmol of sodium citrate per mg of transferrin. These concentrations were selected to achieve a theoretical 100% saturation of the transferrin with iron, assuming a molecular weight of 80,000 for the transferrin (1). FeTf preparations were then dialyzed against 40 mM tris (hydroxymethyl) aminomethane-hydrochloride buffer, pH 7.4, containing 2 mM $NaHCO_3$ for 16 h, dialyzed against 0.15 M NaCl, pH 7.0, for 6 h, and filter sterilized as for ApoTf preparations. Experiments with ^{59}Fe added to the initial reaction mixtures revealed that 85 to 90% of the supplied iron remained specifically bound to the dialyzed FeTf preparations (B. E. Holbein, unpublished data).

Samples of the ApoTf and FeTf preparations were examined for their ratios of absorbance at 465 and 280 nm (A_{465}/A_{280} ratios) (13). Total iron-binding capacity, unsaturated iron-binding capacity, bound iron, and percentage of saturation of the preparations were assayed by ^{59}Fe radioassay (10). These assays were necessary to ensure that the iron was bound by the high-affinity sites of the transferrin.

Infection experiments. Mice were injected with 1.0 ml of ApoTf or FeTf just before the injection of approximately 10^4 CFU of *N. meningitidis* strain M1011 or 0.5 ml of 0.15 M NaCl (control). FeTf, up to 35 mg of protein (45.4 μg of Fe) per mouse (2.3 mg of Fe per kg of body weight), was examined for its ability to promote fatal infection as compared with mice given 0.15 M NaCl or 5 mg of Fe-dextran (10, 11).

The influences of 5 mg of FeTf (0.32 mg of Fe per kg) or 5 mg of ApoTf (0.03 mg of Fe per kg) on bacteremia were examined in mice infected with 10^4 CFU of *N. meningitidis* strain M1011. Controls received 0.15 M NaCl in lieu of transferrin. Two mice per group were bled at 3, 6, 9, 12, 18, and 24 h postinjection, and their bloods were examined for *N. meningitidis* as described before (10, 11).

Influence of ApoTf and FeTf on iron-limited growth of *N. meningitidis* in vitro. *N. meningitidis* strain M1011 cultures, grown on Columbia blood agar for 18 h, were used to loop inoculate 50 ml of NDM containing 200 ng of Fe per ml [$Fe(NH_4)_2(SO_4)_2$]. This level of iron was not limiting in the NDM (3). Cultures were incubated at 37°C with shaking (85 rpm) in an atmosphere of 10% CO_2–air for 16 h. The cultures were used to inoculate fresh NDM broths at 1% (vol/vol) inoculum. These included: NDM alone (200 ng of Fe per ml); NDM with 5 μg of EDDA per ml (an iron chelator from which *N. meningitidis* cannot obtain iron [4]); NDM with EDDA and 0.5 mg of FeTf per ml, and NDM with EDDA and 0.5 mg of ApoTf per ml. The cultures were incubated at 37°C with shaking at 85 rpm in an atmosphere of 10% CO_2–air. Growth was assessed in the cultures by measuring their optical densities at 650 nm at intervals between 0 and 7 h.

RESULTS

Influence of transferrin on infection. The apparent importance of plasma transferrin iron levels to the development of *N. meningitidis* infection prompted a study of human FeTf for its ability to support infection in mice. FeTf injected into mice produced fatal infection in a dose-dependent manner (Table 1). Relatively small amounts of Fe promoted fatal infection. Symptoms of infection were similar in mice given sufficient FeTf and in those given Fe-dextran. It had been established that the severity of the overt symptoms of infection correlate well with numbers of bacteria present in the blood, liver, and spleen of infected mice (11). Complete kill of the mice from infection occurred when 17.5 mg of FeTf carrying 22.7 μg of Fe (1.2 mg of Fe per kg) was injected. It is important to note that similar mortalities could be achieved with

TABLE 1. *Influence of FeTf on N. meningitidis strain M1011 infection in mice*

FeTf injected[a]		% Mortality[b]
mg of protein	μg of Fe	
0 (saline control)	0	0
3.5	4.5	20
7.0	9.1	60
17.5	22.7	100
35.0	45.4	100
	45.4 (FeTf control; no bacteria)	0
	5,000 (Fe-dextran)	100
	5,000 (Fe-dextran control; no bacteria)	0

[a] 93% Fe saturated, as determined by ^{59}Fe radioassay; 1.0 ml injected in physiological saline.

[b] Cumulative mortalities at 72 h postinjection in groups of five mice infected with approximately 10^4 CFU of *N. meningitidis* strain M1011. Average results of two experiments.

Fe-dextran only when 5 mg of Fe (250 mg of Fe per kg) had been injected (10, 11). Thus, FeTf appeared to be approximately 200 times more efficient in promoting fatal infection than iron dextran.

A question remained as to what differing roles the iron and the protein of FeTf could play in this enhancement of infection. This question was addressed by preparing ApoTf and FeTf for comparative studies of their abilities to promote bacteremia in the mice. An amount of transferrin equal to 5 mg was selected for these studies, as this was approximately equal to the total amount of circulating transferrin available in the mice.

The characteristics of the ApoTf and FeTf preparations are shown in Table 2, which indicates that the ApoTf was <10% Fe saturated and that the FeTf was >90% Fe saturated. The high A_{465}/A_{280} ratios for the FeTf preparations along with the ^{59}Fe assays (Table 2) revealed that the iron in these preparations was specifically bound to the high-affinity sites on the transferrin molecules. This is an important aspect, as iron bound non-specifically to other portions of the transferrin molecules could be much more labile.

The ApoTf and FeTf preparations differed greatly in their abilities to stimulate bacteremia in the mice (Fig. 1). Bacteremia in the mice given FeTf was both enhanced and prolonged when compared with the controls. Infection in the FeTf-treated mice was still present at 18 and 24 h postinjection, whereas it had disappeared by 18 h in the control mice. However, ApoTf did not alter the course of infection in the mice. The pattern of a rapid infection followed by its rapid disappearance, which was observed in the control and the ApoTf-treated mice, was similar to that which had been reported previously for control mice (10). The pattern of infection in the mice given FeTf (0.32 mg of Fe per kg) was similar to that which had been observed previously when iron dextran (60 mg of Fe per kg) had been used (10). Thus, these results also

TABLE 2. *Characteristics of transferrin preparations*[a]

Prepn	A_{465}/A_{280}	TIBC (μg of Fe/ml)	UIBC (μg of Fe/ml)	Fe (μg of Fe/ml)	% Saturation
ApoTf	0.002	6.84	6.30	0.56	8.2
FeTf	0.054	6.94	0.46	6.48	93.4

[a] ApoTf and FeTf were prepared at 5 mg/ml as described in Materials and Methods. Data are average values obtained with two identically prepared samples for each. Total iron-binding capacity (TIBC), unsaturated iron-binding capacity (UIBC), iron, and percentage of saturation were determined by ^{59}Fe radioassay.

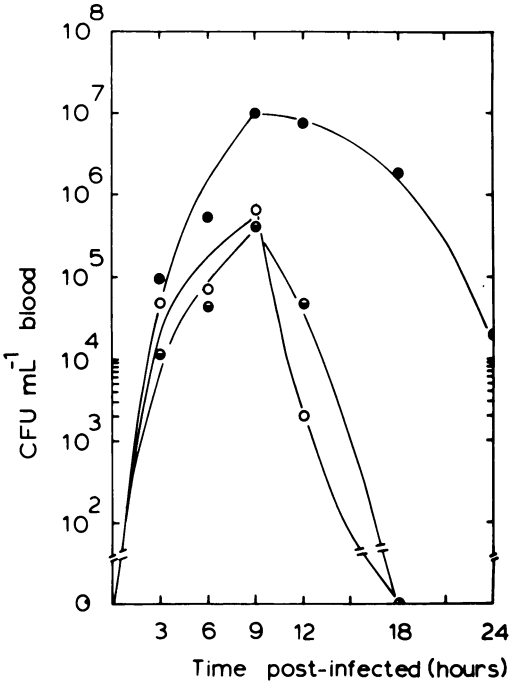

FIG. 1. *Effects of FeTf and ApoTf on N. meningitidis infection in mice. Groups of 25 mice were injected with 5 mg of FeTf (●), 5 mg of ApoTf (◒), or saline (○) just before receiving approximately 10^4 CFU of N. meningitidis strain M1011. Two mice were removed at intervals from each group, and numbers of bacteria in their blood were determined by plate counting. The data represent the average data obtained for three experiments.*

indicated that FeTf was approximately 200 times more efficient in enhancing infection than was iron dextran.

Influence of ApoTf and FeTf on iron-limited growth of *N. meningitidis* in vitro. The ability of FeTf to support the growth of *N. meningitidis* was tested further by examining the abilities of ApoTf and FeTf to relieve EDDA-mediated iron-limited growth in defined medium.

N. meningitidis strain M1011 grew exponentially in NDM containing 200 ng of Fe per ml after a lag phase of approximately 1 h (Fig. 2). However, growth was inhibited when 5 μg of the iron chelator EDDA per ml was included in this medium. The lag phase was extended slightly, and growth was not exponential in the presence of EDDA. The cell yield after 7 h of growth was also substantially less in the EDDA-treated cultures. Thus, it could be concluded that EDDA had produced an iron-limited growth of this bacterium. Transferrin added to cultures containing EDDA was capable of relieving the inhibition of growth. Treatment of iron-limited cultures with FeTf abolished the lag phase, and cell yields after 7 h of growth were similar to those in the control cultures. Interestingly, ApoTf also relieved iron-limited growth. Growth in the ApoTf-treated cultures was similar to growth in the FeTf-treated cultures except that growth leveled off after approximately 5 h and cell yields after 7 h of growth were lower than for the controls (Fig. 2).

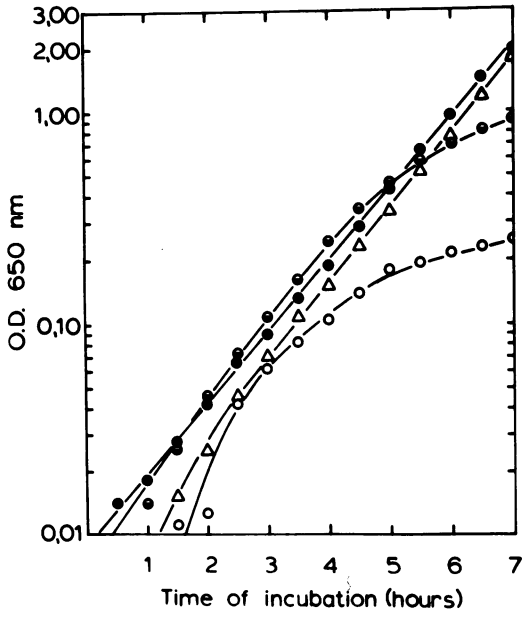

FIG. 2. *Effects of FeTf and ApoTf on EDDA-mediated iron-limited growth of N. meningitidis in vitro. Sixteen-hour cultures of N. meningitidis were used to inoculate (1%, vol/vol) fresh NDM containing 200 ng of Fe per ml (△), NDM with 5 μg of EDDA per ml (○), NDM with EDDA and 0.5 mg of FeTf per ml (●), and NDM with EDDA and 0.5 mg of ApoTf per ml (◒). Growth in the cultures was monitored by measuring their increases in optical density at 650 nm.*

DISCUSSION

This study has shown that small amounts of iron bound specifically to human transferrin were capable of stimulating *N. meningitidis* infection in mice. This finding provides new insights into the importance of iron in this host-parasite relationship. Studies with normal mice (no added iron) had previously indicated that the amount of iron in the transferrin pool was directly related to the course of infection (10). Hypoferremia of the transferrin pool which developed during infection, presumably as a result of host-mediated iron sequestration, correlated with a complete disappearance of infection (10). Iron dextran was also shown to exert its principal effects on the infection by maintaining transferrin iron levels (10). Here, added FeTf maintained the infection in the mice and resulted in fatal infection when sufficient amounts were injected.

Human FeTf was far more efficient at stimulating and prolonging the infection than was iron dextran; 6.5 μg of Fe on transferrin resulted in a similar pattern of infection than had been obtained previously with 1.2 mg of Fe on dextran (10). However, it had been shown that the iron from iron dextran was capable of promoting infection only when it was present in the transferrin pool of the mice, after its initial processing in the reticuloendothelial system. The amount of iron from iron dextran which entered the transferrin pool was indeed small; the bulk of the iron entered the storage pools in the liver and spleen (10). Therefore, it appears that both added iron dextran and human FeTf support infection in a similar manner. The important difference with respect to these two sources of iron is their relative availability as iron sources to *N. meningitidis*. *N. meningitidis* strain M1011 cannot utilize the iron of iron dextran in vitro (5), and therefore iron dextran must be processed by the mice before it is available as iron in the transferrin pool. *N. meningitidis* can, however, utilize the iron on transferrin, as has been shown by the direct acquisition of radiolabeled iron from human transferrin in vitro (4).

Therefore, the marked stimulation of infection by as little as 6.5 μg of Fe suggested that FeTf was an immediate and a preferred source of iron for *N. meningitidis* in vivo. This suggestion was

strengthened by the findings that FeTf or ApoTf could relieve EDDA-mediated, iron-limited growth in a defined medium. EDDA-treated cultures grew as well as the control cultures when supplied with 0.5 mg of FeTf per ml (approximately 650 ng of Fe per ml). Surprisingly, the same amount of ApoTf (approximately 55 ng of Fe per ml) also supported growth. Two possibilities existed to explain these findings. The first was that the ApoTf with its high affinity for Fe [$K_{(ass.)} \cong 10^{36}$ (15)] stripped Fe from the EDDA, thus making it available to the bacteria. The second was that the small amount of Fe associated with the ApoTf was sufficient to provide Fe for the bacteria, without the requirement of the EDDA-associated iron. Growth in the ApoTf-treated cultures leveled off after 5 h, and cell yields at 7 h were lower than those for control or FeTf-treated cultures. The reduced cell yields in ApoTf-treated cultures suggested that iron limitation did occur after substantial growth. This evidence was in support of the second possibility. Archibald and DeVoe (3) have shown that Fe became limiting for chemostat-grown *N. meningitidis* only at levels of <100 ng of Fe per ml. Therefore, the small amounts of FeTf present in the ApoTf preparations could support growth of *N. meningitidis*. The in vitro results demonstrated that the Fe of human transferrin was readily available to *N. meningitidis*, in agreement with the earlier findings of Archibald and DeVoe (4).

The mechanism for the acquisition of Fe from transferrin by *N. meningitidis* remains obscure. It does appear, however, that this mechanism may be quite different than the presumed enterochelin-mediated mechanism used by *E. coli* and *S. typhimurium* (9, 12, 16). *N. meningitidis* did not appear to elaborate a soluble extracellular siderophore when it was examined for its ability to acquire iron from a variety of sources in vitro (5). The acquisition of Fe from human FeTf was also found to require direct cellular association with the transferrin (4).

The possibility of a mechanism for the direct acquisition of iron from transferrin on the surface of the bacterial cell, without the participation of an extracellular siderophore, has important implications for iron acquisition during infection. Plasma concentrations of transferrin range between 2 to 4 mg per ml for humans and other animals (15). The human transferrin pool is typically 25% saturated (8), which corresponds to a transferrin-associated plasma iron concentration of approximately 1,000 ng/ml. This is far in excess of the minimal Fe requirements of *N. meningitidis*. The available iron in the mouse transferrin pool is even higher because its transferrin pool is typically 40 to 50% saturated with iron (10). Thus, a specific mechanism for iron acquisition from transferrin would ensure an adequate supply of iron to *N. meningitidis* during the initial stages of systemic infection of human beings or mice. However, the dependence on a specific mechanism for iron removal from transferrin could also result in an inhibition of growth when the host mobilizes its iron from the transferrin pool during infection. This situation appears to apply during experimental infection of mice. All the iron of the transferrin pool disappeared by 18 h of infection in normal mice (10). The stimulation and maintenance of infection by exogenous FeTf support the hypothesis (10) that the transferrin iron pool of the mice is required for *N. meningitidis* infection. However, direct evidence for the role of mouse transferrin in *N. meningitidis* infection has not been obtained. Conclusive evidence for the role of mouse transferrin during experimental meningococcal infection awaits the availability of sufficient purified mouse transferrin for studies of its roles in iron supply to *N. meningitidis* both in vivo and in vitro.

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