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Comparison of the Abilities of Different Protein Sources of Iron To Enhance *Neisseria meningitidis* Infection in Mice

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This study was done primarily to determine whether the previously observed specificity of the meningococcal transferrin and lactoferrin receptors for human proteins was maintained in vivo during meningococcal infection in mice. Preliminary experiments evaluating the choice of host strain, the age and sex of mice, and the growth conditions of the meningococci indicated that 45-day-old female Swiss Webster mice challenged with meningococci grown on low-pH, low-iron Mueller-Hinton agar plates were appropriate for this study. The comparison of transferrins and lactoferrins from different species demonstrated that only the human forms of these proteins were utilized by meningococci; there was significantly greater mortality among mice treated with iron-saturated human transferrin or lactoferrin (93 and 100%, respectively) than among those not treated or treated with iron-saturated bovine transferrin or bovine lactoferrin (0%). Provision of exogenous hemoglobin also resulted in increased mortality, although not as great as that observed with amounts of transferrin with equivalent iron content, which parallels the more effective utilization of transferrin and lactoferrin in in vitro growth experiments. In addition, unlike with transferrin and lactoferrin, there was no difference in utilization of human and bovine hemoglobin in vitro or in vivo.

Iron is essential for the growth of nearly all microorganisms, and thus specific mechanisms for acquisition of this important element are present in most microbes (22). The human host provides an environment in which the availability of iron is limited. Iron is sequestered by the protein transferrin in the systemic circulation and extracellular compartment (4) and by the protein lactoferrin on mucosal surfaces (12). Although hemoglobin accounts for a large proportion of the total iron in the body, its intracellular location makes it unavailable to most invading microorganisms unless erythrocyte lysis occurs. Released hemoglobin is quickly complexed by the serum protein haptoglobin (6), and similarly, released heme is complexed by the serum protein hemopexin (16).

Neisseria meningitidis is capable of acquiring iron from human transferrin (hTf) (14) and human lactoferrin (hLf) (15) for growth in vitro. The uptake of iron from transferrin requires contact of transferrin with the cell surface, does not result in intracellular accumulation of transferrin, and requires energy (1, 20). The expectation that iron acquisition from these proteins is a receptor-mediated process has been supported by the detection of distinct cell surface receptors for hTf (18) and hLf (19) and the correlation of mutational loss of transferrin-binding activity with loss of ability to specifically utilize transferrin (21). *N. meningitidis* is also capable of acquiring iron from hemoglobin for growth, even when hemoglobin is complexed to haptoglobin (5), but relatively little is known about the mechanism of iron acquisition from hemoglobin. Although *N. meningitidis* can utilize released hemoglobin, it is not known to produce specific hemolysins that would facilitate hemoglobin release.

In previous studies we have demonstrated that the surface receptors for transferrin and lactoferrin have a strong specificity for binding the human forms of these proteins (18, 19). The finding of specificity of receptor binding was complemented by the observation that hTf, but not bovine transferrin (bTf) (18), and hLf, but not bovine lactoferrin (bLf) (19), could support the growth of iron-restricted meningococci in vitro. The demonstration that exogenous hTf increased the level of bacteremia and mortality in a mouse model of meningococcal infection (8) suggests that meningococci were acquiring iron for growth from hTf in vivo. However, these experiments did not allow us to determine whether iron acquisition from transferrin in the mouse was predominantly by a receptor-mediated pathway. In this study we compared the abilities of exogenous human and bovine forms of transferrin and lactoferrin to enhance meningococcal infection in mice in order to determine whether iron acquisition in vivo reflects the observed receptor specificity. We also evaluated human hemoglobin (hHb) and bovine hemoglobin (bHb) as iron sources in vitro and in vivo to determine whether there is a specificity for the human protein and to determine their relative efficacies as iron sources.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N meningitidis* B16B6 and M1011 are standard serotyping strains and were provided by C. Frasch. Strains FAM30, FAM33, and FAM38 are group C, type 2a strains that are wild type, a transferrin iron uptake-deficient mutant, and a transferrin iron uptake-deficient transformant, respectively. FAM30, FAM33, and FAM38 were obtained from P. Frederick Sparling and David W. Dyer. For each experiment, meningococci stored in 30% glycerol at −70°C were streaked onto chocolate agar plates supplemented with CVA enrichment (GIBCO Laboratories, Grand Island, N.Y.) and grown at 37°C in an atmosphere containing 5% CO_2. Cells suspended after growth on chocolate plates that had been incubated for 16 to 24 h were used to inoculate the broth or plate cultures used for the preparation of bacteria for infection or expression experiments. Mueller-Hinton broth (MHB) cultures were inoculated to a starting A_{600} of 0.04 and were incubated with shaking at 37°C for 12 to 16 h before harvest. Mueller-Hinton agar (MHA) plates were inoculated by spreading 50 μl of a heavy suspension of cells (A_{600} between 18 and 30)

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and were incubated at 37°C in an atmosphere containing 5% CO_2 for 16 h before harvest. Low-pH MHB medium containing 50 mM Tris-maleate was prepared as described previously (2). Iron-limited medium was obtained by addition of EDDA (ethylenediamine di-*ortho*-hydroxyphenylacetic acid) to the concentrations indicated below followed by readjustment of the pH. Unless otherwise indicated, the meningococci used in infection experiments were grown on low-pH MHA plates containing 20 μM EDDA. In the growth experiments meningococci were initially grown for 16 h in MHB containing 35 μM EDDA, subcultured to a starting A_{600} of 0.15 into MHB containing 35 μM EDDA for an additional 2-h incubation, and then used to inoculate MHB containing 60 μM EDDA plus the indicated concentrations of iron sources.

Chemicals. Peroxidase-conjugated hTf and hLf were obtained from Jackson Immunoresearch Laboratories, Avondale, Pa. hTf, bTf, hLf, bLf, hHb, and bHb were from Sigma Chemical Co., St. Louis, Mo. Iron dextran was from Dextran Products, Scarborough, Canada. The acrylamide gel exclusion column was from Beckman Instruments, Fullerton, Calif.

Preparation of iron-binding proteins. Iron saturation of transferrins and lactoferrins was achieved as described previously (19). Commercial preparations of iron-saturated proteins were solubilized in a small volume of normal saline and either used directly after filter sterilization or first passed through an acrylamide gel filtration column to remove free iron. Protein preparations were concentrated by ultrafiltration with a Centriflo membrane cone (Amicon Corp., Danvers, Mass.) before sterile filtration through a 0.2-μm membrane. Solutions of hemoglobin were prepared by solubilization directly in normal saline. After centrifugation at 13,000 × *g* for 5 min to remove insoluble debris, the supernatant was filter sterilized as described above. The protein contents of the sterile solutions were estimated by the method of Lowry et al. (10) with bovine serum albumin as the standard. The iron contents of the protein solutions were calculated by assuming 100% saturation of the proteins, which was verified for transferrin and lactoferrin by measurement of the A_{465} (13).

Transferrin- and lactoferrin-binding assay. Cells collected by centrifugation from broth cultures or directly from plates were suspended in normal saline to an A_{600} of 5 and diluted as indicated. Two-microliter portions of the cell suspensions were spotted onto nitrocellulose-cellulose acetate membranes and assayed for transferrin- and lactoferrin-binding activities essentially as described previously (18).

Mouse infection experiments. Swiss Webster and C57BL/6 mice were obtained from Charles River Laboratories, Montreal, Canada. They were given food and water ad libitum throughout all experiments. Suspensions of meningococci in 0.1 ml of MHB were administered by intraperitoneal (i.p.) injection. The challenge dose was initially estimated from the observed A_{600} of the cell suspensions but was confirmed by plating 10-fold serial dilutions of the challenge suspension on chocolate plates. Control groups of mice received either no additional treatment or an i.p. injection of 31 mg of iron dextran in 0.5 ml of normal saline immediately before challenge. In preliminary experiments no mortality was observed in unchallenged mice at the dose of iron dextran used. In the test mice, 0.5-ml portions of normal saline containing various amounts of protein were administered i.p. just before challenge, and where indicated, additional 0.2-ml portions of protein solution were administered intravenously (i.v.) via the tail vein at various times. The calculations of the 50% endpoints (17) and the cumulative mortalities were performed 72 h after challenge. Statistical comparisons of mortalities were made using the statistical calculator for two-by-two and two-by-*n* tables in the Epiinfo software package. To verify the validity of the calculated *P* values, they were compared with *P* values calculated with Fisher's exact test by using the Epistat software program.

RESULTS

Selection of the mouse model of infection. Several studies on meningococcal infections in mice have demonstrated the importance of the host mouse strain (3, 23), the meningococcal challenge strain (3, 9), and the growth conditions for meningococci before challenge (2). In addition, the sex and age of mice used in published meningococcal-infection experiments varied, and the effect of these parameters was uncertain. Thus, before selection of the appropriate conditions for our mouse infection experiments, we briefly evaluated the effect of these parameters. Initially we determined the 50% lethal doses (LD_{50}s) of strains B16B6 and M1011 in female C57BL/6 mice (LD_{50}s, 6×10^3 and 4×10^4, respectively) and Swiss Webster mice (LD_{50}s, 5×10^3 and 5×10^2, respectively), using iron dextran treatment to increase the susceptibility of the mice. The results demonstrated that both of these meningococcal strains are reasonably virulent in both the outbred Swiss Webster mice and the inbred C57BL/6 mice. Meningococcal strain B16B6 was ultimately chosen since all previous growth studies with transferrin and lactoferrin and previous receptor studies were performed with this strain (18, 19). Meningococcal strains FAM30, FAM33, and FAM38, which include mutants specifically deficient in transferrin iron acquisition (21), were also tested for virulence in Swiss Webster and C57BL/6 mice in similar experiments with smaller numbers of mice. No deaths occurred with challenges of up to 10^6 bacteria, indicating that these strains were relatively avirulent and could not be incorporated into further studies. An experiment designed to evaluate the effect of sex and age of mice was performed with groups of four male or female Swiss Webster mice ranging in age from 25 to 45 days and challenged with between 10 and 10^6 iron-starved meningococci. The LD_{50} was lowest for the group of 45-day-old female mice, and thus all subsequent experiments were performed with female Swiss Webster mice of this age.

Since the objective of this study was to compare the abilities of different protein sources of iron to enhance meningococcal infection in mice, it was presumed to be important to use meningococci which were expressing components necessary for iron acquisition. Previous studies have demonstrated that growth under iron-limiting conditions is necessary for expression of transferrin iron acquisition ability (20) and for expression of transferrin-binding ability (18). Therefore, we used a simple dot binding assay (18, 19) to evaluate the levels of transferrin- and lactoferrin-binding activities in cells grown under different conditions and assumed that high levels of receptor activity correlated with proficiency in iron acquisition pathways. With this type of analysis, we determined the level of the specific iron chelator EDDA to be added to different growth media in order to obtain both optimum expression of activity and reasonable growth yield. Using the inoculum and growth conditions described in Materials and Methods, we determined that the optimum level of EDDA added to MHB was 30 μM and that the optimum level of EDDA added to MHA plates was 20 μM.

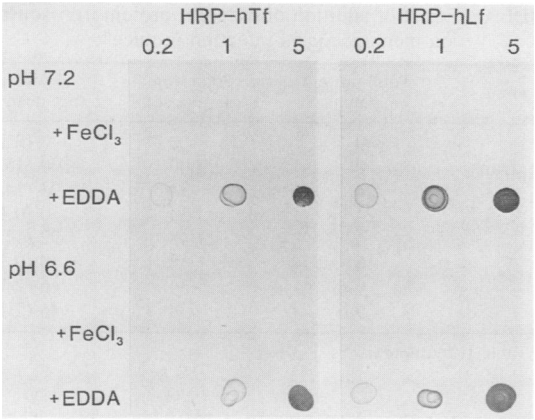

FIG. 1. Effect of pH and EDDA on expression of transferrin- and lactoferrin-binding activities. Meningococcal strain B16B6 freshly grown on chocolate plates was used to inoculate (A_{600}, 0.05) overnight broth cultures of buffered MHB at pH 6.6 or 7.2 with or without added EDDA (30 μM). The overnight cultures were harvested by centrifugation, suspended to a final A_{600} of 5, and, after preparation of dilutions, spotted onto HA paper and assayed for transferrin- or lactoferrin-binding activity as described in Materials and Methods. HRP-, Horseradish peroxidase conjugated. Numbers at the top indicate the A_{600}s of cell suspensions spotted onto paper. $FeCl_3$ was added to 45 μM where indicated.

In view of the reported effect of pH of the growth medium on virulence of meningococci (2), we analyzed the effect of pH on expression of receptor activity. Growth of meningococci in pH 7.2 or 6.6 buffered MHB without additions, or with addition of excess $FeCl_3$, resulted in virtually no detectable transferrin- or lactoferrin-binding activity (Fig. 1). Addition of EDDA resulted in a greater than 25-fold increase in both binding activities and achieved essentially the same level of binding activities in the two broths (pH 7.2 and 6.6). Thus, the pH of the broth does not appear to independently or synergistically affect receptor expression.

Although receptor expression was anticipated to be important for subsequent infection experiments it was necessary to verify that growth conditions selected for maximal receptor expression did not jeopardize the virulence of the meningococci. An experiment was performed to compare the virulence of meningococci grown under various conditions. When we compared meningococci grown in low-pH MHB (containing 30 μM EDDA) with cells grown on low-pH MHA plates (containing 20 μM EDDA) in the mouse infection model, the latter cells were more virulent (LD_{50}s, 4×10^5 and 6×10^3, respectively). Meningococci grown in broth had similar levels of transferrin- and lactoferrin-binding activities as did the meningococci obtained from plate cultures. When cells suspended after growth on chocolate plates were used in this experiment, the resulting LD_{50} was 9×10^4, indicating that low pH or iron limitation (or both) resulted in an increased virulence, as observed previously (2,11).

Administration of iron-containing proteins. The original experiments demonstrating the effect of supply of exogenous hTf on meningococcal infection in mice utilized i.p. injection of transferrin just before i.p. challenge (8). This study also demonstrated that after i.p. challenge significant bacteremia developed within 3 h. We were uncertain whether the hTf supplied i.p. would be available to the bacteria invading the vascular compartment, and thus we decided to evaluate the effect of route of administration of hTf. In the study outlined in Table 1, a standard dose of hTf either was administered by a single i.p. injection or was divided between i.p. and i.v. injections. Mice receiving a single dose of hTf by i.p. injection were also given a control i.v. injection of saline 4 h postchallenge. In this experiment, the highest level of mortality was observed in groups of mice receiving the combined regimen of hTf administration, but the differences between the group treated with a single i.p. injection of hTf and the groups treated with two injections were not statistically significant ($P > 0.5$, Yates corrected). Nevertheless, we selected the combined regimen of hTf administration for subsequent experiments.

TABLE 1. Effect of route of administration of hTf[a]

Iron source and mouse group	Amt of protein administered (mg/mouse)		Total iron (μg/mouse)	% Mortality (no. dead/total)
	i.p.	i.v.		
None	0	0	0	0 (0/15)
Iron dextran	0	0	9,000	100 (18/18)
hTf				
1	8	0	21	44 (7/16)
2	4	4	21	60 (9/15)
3	6	2	21	60 (9/15)

[a] Groups of five female Swiss Webster mice received an i.p. injection of 0.5 ml of saline containing the indicated amounts of iron supplement just before challenge and an i.v. injection of 0.2 ml of saline containing the indicated amounts of iron supplement 4 h postchallenge. Iron dextran was given as a single i.p. dose just before challenge. The mice were challenged by an i.p. injection of 10^7 iron-starved strain B16B6 meningococci in 0.1 ml of MHB, and mortality was determined 72 h postchallenge.

In order to compare the effects of human and nonhuman proteins, it was important to initially determine the amounts of exogenous hTf, hLf, and hHb resulting in high levels of mortality. High levels of mortality were achieved with doses of exogenous hTf and hLf as low as 6 mg of protein (8 μg of iron) per mouse, but higher doses of hHb were required (19.2 mg of protein [64 μg of iron] per mouse) to achieve similar mortality rates (Table 2). To complement the proposed mouse infection model experiment comparing the effects of human and bovine proteins, we established an in vitro

TABLE 2. Effect of dose of hTf, hLf, or hHb on mortality[a]

Iron source and mouse group	Amt of protein administered (mg/mouse)		Total iron (μg/mouse)	% Mortality (no. dead/total)
	i.p.	i.v.		
None	0	0	0	0 (0/5)
Iron dextran	0	0	9,000	100 (5/5)
hTf				
1	4.5	1.5	8	80 (4/5)
2	9.0	3.0	16	80 (4/5)
3	18.0	6.0	32	100 (5/5)
4	36.0	12.0	64	100 (5/5)
hLf				
1	1.5	0.5	2.7	10 (1/10)
2	4.5	1.5	8	90 (9/10)
3	18.0	6.0	32	100 (4/4)
hHb				
1	1.8	0.6	8	0 (0/5)
2	3.6	1.2	16	0 (0/5)
3	7.2	2.4	32	40 (2/5)
4	14.4	4.8	64	80 (4/5)

[a] See Table 1, footnote *a*.

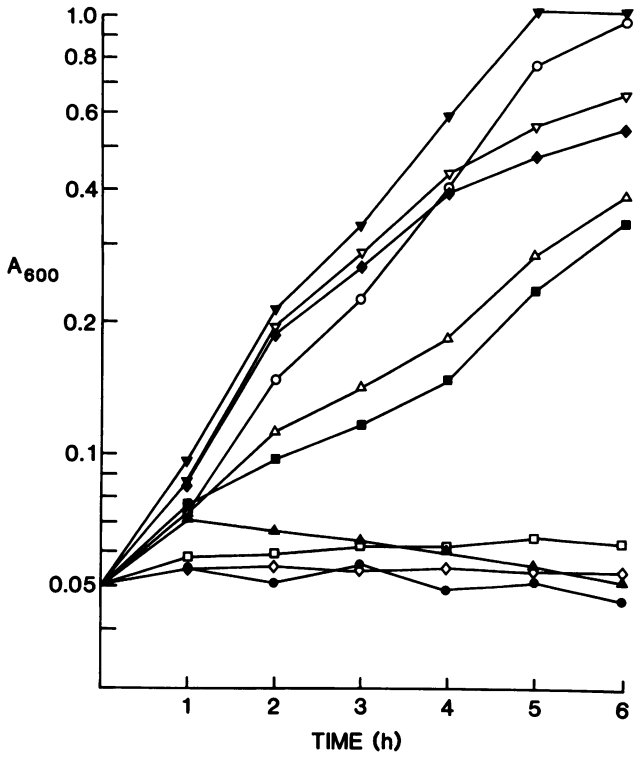

FIG. 2. Utilization of different iron sources for growth of iron-deficient strain B16B6. Strain B16B6 was rendered iron deficient by overnight growth and a 2-h incubation in iron-deficient medium as described in Materials and Methods. Iron-deficient meningococci were used to inoculate different flasks containing MHB medium with 60 μM EDDA plus the indicated added iron source, and the flasks were monitored for growth for a period of 6 h. Symbols: ◇, no addition; ○, 75 μM $FeCl_3$; ◆, 1 μM hTf; ●, 1 μM bTf; ▽, 1 μM hLf; ▼, 10 μM hLf; □, 1 μM bLf; ▲, 10 μM bLf; △, 0.5 μM hHb; ■, 0.5 μM bHb.

growth experiment to compare these various iron sources in vitro. Iron-starved meningococci were able to effectively use 1 μM hTf or hLf but were incapable of using the bovine forms of these proteins (Fig. 2). In contrast, iron-starved meningococci were capable of using either hHb or bHb, but the amount of growth attained with hemoglobins was less than that achieved with hTf or hLf containing similar quantities of iron. The prior observation that bLf appeared to competitively bind to the lactoferrin receptor at high concentrations (19) was of some concern, since the planned mouse infection model experiments would provide proteins at relatively high i.p. protein concentrations. Therefore, we also evaluated in in vitro growth experiments whether the specificity for hLf would be maintained at higher protein concentrations. Meningococci were incapable of utilizing bLf at even 10 μM concentrations (Fig. 2).

To determine whether the specificity observed in vitro would be maintained in vivo, experiments were established comparing the abilities of different exogenous protein sources of iron to increase the mortality of meningococcal infection in mice. *N. meningitidis* B16B6 was able to use hTf and hLf but not bTf or bLf as a source of iron in vivo (Table 3). A highly significant difference in mortality was observed ($P << 0.001$) when untreated mice were compared with mice treated with hTf or hLf. Similarly, there was a highly significant difference ($P << 0.001$) between the mortality

TABLE 3. Effect of addition of different protein iron sources on meningococcal infection in mice[a]

Iron source	Total protein (mg/mouse)	Total iron (μg/mouse)	% Mortality (no. dead/total)
None	0	0	0 (0/15)
Iron dextran	0	9,000	100 (15/15)
hTf	24	34	93 (14/15)
bTf	24	34	0 (0/15)
hLf	6	8	92 (11/12)
bLf	6	8	0 (0/12)
hHb	9.6	34	46 (7/15)
bHb	9.6	34	46 (7/15)

[a] See Table 1, footnote *a*.

observed with hTf- or hLf-treated mice and that observed with mice treated with the bovine proteins.

Table 3 also illustrates that provision of hHb or bHb in quantities containing amounts of iron equivalent to that in the exogenously supplied hTf resulted in a significant increase in mortality over that of control mice ($P < 0.01$). In contrast to the transferrins and lactoferrins, there was no significant difference in the mortalities ($P > 0.5$) obtained with hHb and bHb. However, this level of mortality was substantially less than that observed in hTf-treated mice ($P < 0.05$), which correlates with the results in the in vitro growth studies. A comparison of the results in Table 3 and Fig. 2 indicates that the relative efficacies of the various proteins sources of iron to supply iron for growth were similar in vitro and in vivo.

DISCUSSION

Previous studies have demonstrated that growth under low-pH and low-iron conditions increased the virulence of meningococci in iron dextran-treated mice (2, 11). They also showed that growth under low-pH but iron-sufficient conditions, or growth under normal-pH but iron-deficient conditions, significantly lowered the LD_{50} but that growth under both low-pH and iron-deficient conditions resulted in the greatest overall decrease in the LD_{50}. Our observation tht transferrin and lactoferrin receptors are not significantly expressed in meningococci grown under low-pH and iron-sufficient conditions (Fig. 1) correlates with the previous demonstration that iron acquisition from transferrin is virtually absent in these cells (20) and supports the hypothesis that factors not involved in iron acquisition, such as capsule, are responsible for the observed increase in virulence at low pH (11). The effect of iron starvation on virulence of meningococci in the iron dextran-treated mouse model may also be unrelated to expression of factors involved in iron acquisition. It has been shown that growth under iron-deficient conditions also results in an increase in the level of capsular polysaccharide and that growth under low-pH, low-iron conditions results in production of capsular polysaccharide levels higher than those observed with low pH or low iron alone, which correlates with the observed increases in virulence (11).

Although there may not be any conclusive evidence that expression of meningococcal components involved in iron acquisition results in increased virulence in animal infection models, this does not preclude such components from being essential for infections in humans. First, the commonly utilized method of administration of iron dextran to increase the susceptibility of mice to bacterial infection impairs the ability of the host to limit the availability of iron to the

bacteria (7, 9). Thus, the requirement for expression of high-affinity iron acquisition systems would be minimized. Second, expression of high-affinity transferrin and lactoferrin iron acquisition systems that are specific for human proteins (18, 19; Fig. 2 and Table 2) may not provide a strong selective advantage when the transferrin available in the host cannot be utilized (18). Third, the regulation of expression of these components is such that by the time that the availability of iron begins to limit the growth rate of the bacteria, a significant level of expression is attained (18). Thus, obtaining evidence for the role of these components in the pathogenesis of meningococcal infection may require utilization of a mouse infection model with exogenously supplied hTf or hLf and specific meningococcal mutants.

In previous studies (18, 19) we established by competitive binding studies that the transferrin and lactoferrin receptors were specific for the human proteins and also observed this specificity during in vitro growth. These results strongly suggested that under the in vitro conditions utilized, iron acquisition from transferrin and lactoferrin was exclusively occurring via a receptor-mediated iron acquisition pathway. In the present study we have confirmed the specificity for hTf and hLf, even at higher protein concentrations, during in vitro growth and have demonstrated that, in contrast, hHb and bHb are equally effective iron sources for growth (Fig. 1). In addition, with a mouse infection model we have demonstrated that only hTf and hLf could be utilized by meningococci in vivo, resulting in increased mortality in mice treated with these proteins (Table 3). The inability of bTf and bLf to cause increased mortality (or subjective disease symptomatology) indicates that these iron sources were not utilized by meningococci in vivo, which has obvious implications for explanations of the host specificity of natural meningococcal infection and for development of animal models. These results also strongly suggest that utilization of hTf and hLf in the mice was predominantly via the receptor-mediated iron acquisition pathway and that alternate pathways for acquiring iron from the bovine proteins were absent or ineffective. The equal efficacies of hHb and bHb iron sources in the mouse infection model (Table 3) strengthen the correlation between in vitro and in vivo results and make it unlikely that the selectivity for hTf or hLf could be due to a more general phenomenon in which human proteins were preferentially removed or destroyed. Ultimately, the importance of receptor-mediated iron acquisition in vivo should be demonstrated with mutants carrying specific mutations in the receptor-mediated pathway (21), but unfortunately, strains FAM30, FAM33, and FAM38, which we hoped to use for this purpose, were avirulent in the mouse model of infection that we used.

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LITERATURE CITED

1. **Archibald, F. S., and I. W. DeVoe.** 1979. Removal of iron from human transferrin by *Neisseria meningitidis*. FEMS Microbiol. Lett. **6**:159–162.
2. **Brener, D., I. W. DeVoe, and B. E. Holbein.** 1981. Increased virulence of *Neisseria meningitidis* after in vitro iron-limited growth at low pH. Infect. Immun. **33**:59–66.
3. **Brodeur, B. R., P. S. Tsang, J. Hamel, Y. Larose, and S. Montplaisir.** 1986. Mouse models of infection for *Neisseria meningitidis* B,2b and *Haemophilus influenzae* type b diseases. Can. J. Microbiol. **32**:33–37.
4. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1974. Bacterial iron metabolism in infection and immunity, p. 518–551. *In* J. B. Neilands (ed.), Microbial iron metabolism: a comprehensive treatise. Academic Press, Inc., New York.
5. **Dyer, D. W., E. P. West, and P. F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic neisseriae with heme-bound iron. Infect. Immun. **55**:2171–2175.
6. **Eaton, J. W., P. Brandt, J. R. Mahoney, and J. T. Lee, Jr.** 1982. Haptoglobin: a natural bacteriostat. Science **215**:691–693.
7. **Holbein, B. E.** 1980. Iron-controlled infection with *Neisseria meningitidis* in mice. Infect. Immun. **29**:886–891.
8. **Holbein, B. E.** 1981. Enhancement of *Neisseria meningitidis* infection in mice by the addition of iron bound to transferrin. Infect. Immun. **34**:120–125.
9. **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.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. **Masson, L., and B. E. Holbein.** 1985. Influence of nutrient limitation and low pH on serogroup B *Neisseria meningitidis* capsular polysaccharide levels: correlation with virulence for mice. Infect. Immun. **47**:465–471.
12. **Masson, P. L., J. F. Heremans, and C. H. Dive.** 1966. An iron-binding protein common to many external secretions. Clin. Chim. Acta **14**:729–734.
13. **Mazurier, J., and G. Spik.** 1980. Comparative study of the iron-binding properties of human transferrins. I. Complete and sequential iron saturation and desaturation of the lactotransferrin. Biochim. Biophys. Acta **629**:399–408.
14. **Mickelsen, P. A., E. Blackman, and F. P. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meninigitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. Infect. Immun. **33**:555–564.
15. **Mickelsen, P. A., E. Blackman and F. P. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meninigitidis*, and commensal *Neisseria* species to obtain iron from lactoferrin. Infect. Immun. **35**:915–920.
16. **Muller-Eberhard, U.** 1970. Hemopexin. N. Engl. J. Med. **283**: 1090–1094.
17. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
18. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the transferrin receptor from *Neisseria meninigitidis*. Mol. Microbiol. **2**:281–288.
19. **Schryvers, A. B., and L. J. Morris.** 1988. Identification and characterization of the lactoferrin-binding protein from *Neisseria meningitidis*. Infect. Immun. **56**:1144–1149.
20. **Simonson, C., D. Brener, and I. W. DeVoe.** 1982. Expression of a high-affinity mechanism for acquisition of transferrin iron by *Neisseria meningitidis*. Infect. Immun. **36**:107–113.
21. **Tsai, J., D. W. Dyer, and P. F. Sparling.** 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56**:3132–3138.
22. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
23. **Woods, J. P., J. A. Frelinger, G. Warrack, and J. G. Cannon.** 1988. Mouse genetic locus Lps influences susceptibility to *Neisseria meningitidis* infection. Infect. Immun. **56**:1950–1955.