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Expression of a High-Affinity Mechanism for Acquisition of Transferrin Iron by *Neisseria meningitidis*

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Iron-starved meningococci grown at either pH 7.2 or 6.6 were capable of removing and incorporating iron from human transferrin by a saturable, cell surface mechanism that specifically recognized transferrin rather than iron. The maximum expression of the iron uptake system occurred after 4 h of iron starvation. The uptake of the iron was dependent upon a functioning electron transport chain and was sensitive to 60°C and trypsin. Cells grown under iron-sufficient conditions were incapable of accumulating iron from transferrin. No evidence was found for a primary role for cell-free soluble siderophores in the removal of iron from transferrin. The nonpathogenic neisseriae, *Neisseria flava* and *N. sicca*, were unable to utilize iron on transferrin.

The requirement for iron in the initiation and maintenance of bacterial infections is well documented (9, 22). Although vertebrate hosts possess large quantities of iron, the only source accessible to microorganisms invading the systemic circulation is the serum glycoprotein transferrin. As a result of the extremely high affinity of transferrin for iron, the concentration of free iron in serum is 10^{-18} M (9), far below that necessary to sustain bacterial growth. Therefore, the ability of an invading pathogen to compete successfully for transferrin-bound iron has been considered an important virulence factor (14).

In response to low iron in their environment, many microorganisms excrete highly specific iron chelators (siderophores) with affinities for iron comparable to that of transferrin (22). Archibald and DeVoe (6) have shown that the growth of the meningococcus with transferrin as the sole source of iron depends on surface contact between the meningococcal cell and the transferrin molecule. When the transferrin was separated from the meningococcus by a dialysis membrane with a 12,000-dalton exclusion limit, the meningococci were incapable of growth.

In a recent report from this laboratory, Brener et al. (8) showed that iron starvation and low pH during growth greatly enhanced the virulence of meningococci for mice. Here we present evidence for a meningococcal surface mechanism for the removal of iron from transferrin. The iron uptake mechanism was expressed in response to iron starvation and was enhanced during growth at low culture pH. Iron uptake initially depended upon the specific recognition of transferrin at the cell surface rather than the iron on this glycoprotein. The uptake of iron from the transferrin required a functional respiratory system.

MATERIALS AND METHODS

Organism. All stock cultures of the group B *Neisseria meningitidis* SD1C used in these experiments were those of the smooth colonial type (11). *Neisseria sicca* 19 stock culture maintenance was described previously (15). *Neisseria flava* (M-953) was obtained from the neisseria repository, NAMRU, School of Public Health, University of California, Berkeley, and stock cultures were maintained on Mueller-Hinton (MH; Difco Laboratories, Detroit, Mich.) slants at −70°C. The routine maintenance of meningococcal stock cultures and the procedures for strain purity checks were described previously (10).

Cell growth and preparation. Microorganisms were grown in MH liquid medium (Difco) or on the same medium with the addition of 1.5% agar. Cultures on plates were incubated in a candle-extinction jar at 37°C (100% relative humidity) for 18 h. Broth media were prepared with or without the addition of the iron chelator ethylenediamine di-ortho-hydroxyphenylacetate (EDDA; ICN Pharmaceuticals Inc., Plainview, N.Y.) to deprive the meningococcus of available iron in the medium (8). Broth media were buffered with 25 mM Tris-maleate (Sigma Chemical Co., St. Louis, Mo.) and adjusted to pH 6.6 or 7.2 with 4 N NaOH. Cells from an 18-h culture on MH agar were suspended in the MH broth at the appropriate pH with or without EDDA (7 μg/ml); portions of the suspension were used as the inoculum for 100 ml of one of the following four MH liquid media (final optical density at 600 nm, 0.07): (i) pH 7.2 without EDDA; (ii) pH 7.2 with EDDA; (iii) pH 6.6 without EDDA; and (iv) pH 6.6 with EDDA. All broth cultures were incubated with shaking (100 rpm) at 37°C.

Transferrin preparation. Human serum transferrin (>98% purity, personal communication, Sigma) was deferrated by dialysis against two changes of 0.1 M

citrate-acetate buffer (pH 4.5) as described by Aisen et al. (3) followed by dialysis against two changes of distilled water. The preparation was then dialyzed against 20 mM sodium bicarbonate in 40 mM Tris buffer (Trizma base; Sigma), pH 7.4, to provide the bicarbonate ions required for the binding of iron (2, 4). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the transferrin, a single band (80,000 daltons) appeared upon staining with Coomassie blue by methods given previously (8).

$^{59}FeCl_3$ (New England Nuclear Corp., Boston, Mass.) was complexed with ^{56}Fe-citrate by using a 10-fold molar excess of sodium citrate and mixed with apotransferrin in the Tris-bicarbonate buffer described above (30 min, 22°C) followed by dialysis (16 h) against the same buffer to remove unbound iron-citrate. To achieve 30% saturation of the transferrin binding sites, 0.42 μg of iron was added per mg of apotransferrin. To fully saturate the transferrin, 1.4 μg of iron as iron-citrate per mg of apotransferrin was used with the same protocol as that described above for 30% saturated transferrin. Apotransferrin was labeled with ^{125}I, when appropriate, by using the protein radioiodination system from New England Nuclear Corp. Iron was loaded onto the transferrin after the iodination process. The iodination procedure did not alter the iron-binding ability of the transferrin molecules.

Uptake of ^{59}Fe-transferrin and Fe-[^{125}I]transferrin. Early stationary-phase cells were harvested by centrifugation (10,000 × *g*, 10 min) and suspended in iron-free (<6 ng of Fe/ml) neisseria defined medium (NDM) (5), pH 7.4, to a final cell concentration of approximately 3×10^9 colony-forming units per ml. After a 15-min equilibration at 37°C, 100 μl of ^{125}I- or ^{59}Fe-labeled, 30% saturated human transferrin, or iron-free apotransferrin (30 μM, final concentration) in NDM was added to 4.0 ml of the cell suspension. The specific activity of ^{59}Fe used for uptake experiments was 70 μCi/μmol. At appropriate time intervals 0.5-ml samples were removed and mixed with 0.5 ml of a 20-fold-excess concentration of 30% saturated transferrin or apotransferrin (600 μM, final concentration) in iron-free NDM to prevent further uptake or binding of either ^{59}Fe- or ^{125}I-labeled transferrin. Cells were collected by centrifugation (15,000 × *g*, 1 min; Microcentrifuge, Eppendorf) and washed twice by suspension in and centrifugation from iron-free NDM. Radioactivity of samples was determined with a Beckman 8000 gamma counter (Beckman Instruments Inc., Fullerton, Calif.).

Assay for soluble siderophore. ^{59}Fe-transferrin was immobilized on cyanogen bromide-linked Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) to determine whether spent NDM culture supernatant fractions contained excreted meningococcal siderophores which would remove ^{59}Fe from the transferrin. Procedures for preparing, washing, and coupling the transferrin to the gel are described elsewhere (18). Cell pellets of *N. meningitidis* SD1C from an iron-limited broth culture in MH-EDDA (pH 7.2 or 6.6) were suspended in iron-free NDM and incubated (37°C). After 60 min cells were removed by centrifugation (10,000 × *g*, 10 min), and the supernatant fluids were centrifuged at high speed (70,000 × *g*, 60 min; 60 Ti rotor, Beckman L5-6B Ultracentrifuge) to remove any outer membrane blebs released from cells during incubation. The transferrin-Sepharose gel was suspended in the supernatant fluid from the high-speed centrifugation. After 60 min the supernatant fraction was analyzed for radioactivity released from the transferrin.

Inhibitors of electron transport. The source, methods of application, and modes of action of KCN, NaN_3, and antimycin A were described by Yu and DeVoe (24).

Treatment of glassware. All glassware was washed with 6 N HCl and thoroughly rinsed in deionized, distilled water before use.

RESULTS

Meningococci in iron-free NDM were given ^{59}Fe-transferrin, but only those cells previously starved for iron were capable of removing iron from this glycoprotein (Fig. 1). This ability was independent of the pH at which cells had been grown. During the first 5 min the rate of iron accumulation was similar for iron-starved cells grown at either pH 6.6 or 7.2. Cells starved for iron during growth at pH 7.2 initially took up iron rapidly in NDM, but appeared near saturation after 15 min, whereas iron-starved cells from pH 6.6 cultures continued to accumulate progressively more iron over the course of the experiment. After 60 min, the iron associated with the cells grown at pH 6.6 represented approximately 30% of the total available iron on transferrin. This continued uptake, although at a

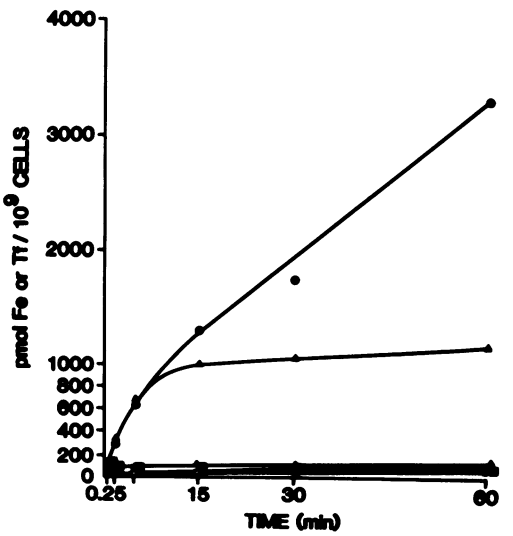

FIG. 1. Uptake of iron from ^{59}Fe-transferrin or the binding of Fe-[^{125}I]transferrin by *N. meningitidis* cultured in MH medium at low or neutral pH, with or without the addition of EDDA. Symbols: (●) ^{59}Fe-transferrin, pH 6.6, MH-EDDA; (▲) ^{59}Fe-transferrin, pH 7.2, MH-EDDA; (△) Fe-[^{125}I]transferrin, pH 7.2, MH-EDDA; (□) Fe-[^{125}I]transferrin, pH 6.6, MH-EDDA; (○) ^{59}Fe-transferrin, pH 6.6, MH; (■) ^{59}Fe-transferrin, pH 7.2, MH.

constant rate, was slower than that over the first 5 min. The distribution of the iron within the cell (pH 6.6 grown) was determined after breaking cells in a French press. Approximately 40% of the iron was sedimentable (60 min, 70,000 × *g*), and the distribution in the envelope was approximately 70% in the inner membrane and 22% in the outer membrane (Simonson and DeVoe, unpublished data). In contrast to iron-starved cells, meningococci cultured in iron-sufficient medium were completely ineffective at removal and uptake of iron from transferrin (Fig. 1). Nonpathogenic neisseriae, *N. sicca* and *N. flava*, were also unable to mobilize the iron from transferrin, despite prior iron starvation (Fig. 2). Such results are in agreement with those of Mickelsen and Sparling (16).

The expression of the iron uptake system increased with the time of iron deprivation (Fig. 3). Maximum iron uptake rates were observed after incubation of the meningococci in iron-limited medium for 4 h. After 5 h the bacterial viability, and hence the uptake of ^{59}Fe, declined (Fig. 3, inset).

During the 1-h uptake experiment (Fig. 1), cells that were no longer able to grow in MH-EDDA broth because of severe iron limitation underwent slightly more than one doubling when suspended in Fe-transferrin-NDM (results in Fig. 1 are calculated on the basis of cell numbers, which were monitored during the experiments). The renewed growth in the iron-deprived cell populations is evidence that the iron

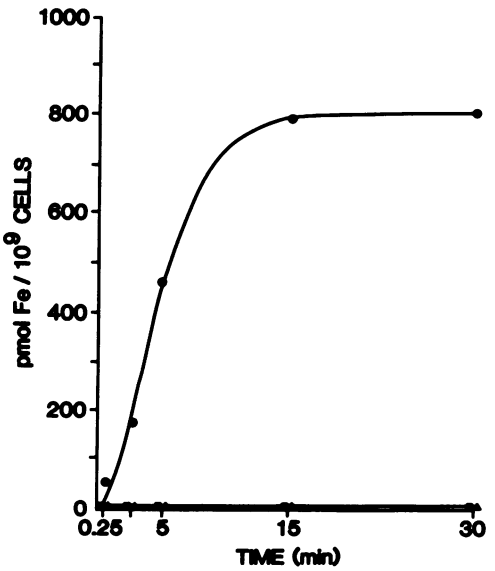

FIG. 2. Uptake of iron from ^{59}Fe-transferrin by *N. meningitidis* (●), *N. sicca* (■), and *N. flava* (▲) grown in MH-EDDA (pH 7.2).

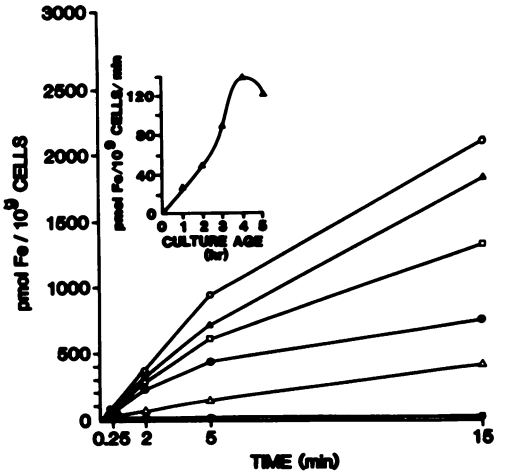

FIG. 3. Uptake of iron from ^{59}Fe-transferrin (30 μM; 30% saturated) by *N. meningitidis* grown in MH or MH-EDDA medium at pH 7.2 for various lengths of time. Symbols: (■) MH-grown cells; MH-EDDA-grown cells 1 h (△), 2 h (●), 3 h (□), 4 h (○), and 5 h (▲) postinoculation. Inset, Initial rates of uptake of iron from ^{59}Fe-transferrin by iron-limited *N. meningitidis* versus the time of iron deprivation.

was not only taken up by the cells but also metabolized.

Similar findings were obtained by Archibald and DeVoe (7) who gave iron-starved meningococci in NDM Fe-transferrin as a sole source of iron; cells responded within minutes by initiating cell division. In spite of the initiation of cell division, it could be argued that the ^{59}Fe uptake by the cells was merely the result of increased binding of the transferrin to the cell surface with time. If such were the case, one could expect the amount of cell-associated iron to be in direct proportion to the transferrin. As the transferrin was 30% iron saturated (approximately that of normal serum) (22), one could expect a maximum iron/transferrin ratio of 0.6:1.0. The 32:1 ratio obtained after 60 min (Fig. 1) in iron-starved, pH 6.6-grown cells given ^{59}Fe- or ^{125}I-labeled transferrin indicates that iron was indeed removed from transferrin. In contrast, the same ratio for cells from iron-sufficient cultures was in the order of <0.5:1.0, as one would expect if transferrin had been bound but the iron had not been incorporated by the cells.

The enhanced uptake of iron observed in cells cultured at low pH was not attributable to an increased amount of transferrin bound to these cells (Table 1). The number of transferrin-binding sites was similar for cells grown at pH 6.6 or 7.2. Although surface contact between the meningococcal cell surface and the transferrin molecule was essential for the unloading of iron, it is not known whether the measured transferrin

TABLE 1. Binding of apotransferrin and 30% saturated transferrin to *N. meningitidis* cultured in iron-sufficient or iron-limited medium at low or neutral pH

MH medium[a]		pmol of Tf/10^9 cells[b]	Molecules of Tf/cell ($\times 10^4$)	pmol of ApoTf/10^9 cells[b]	Molecules of ApoTf/cell ($\times 10^4$)
pH	EDDA				
6.6	+	71	4.3 ± 0.45[c]	71	4.3 ± 0.06
6.6	−	83	5.1 ± 0.18	87	5.2 ± 1.3
7.2	+	76	4.6 ± 1.9	ND[d]	ND
7.2	−	75	4.5 ± 0.35	71	4.3 ± 0.58

[a] MH broth was buffered with 25 mM Tris-maleate and adjusted to pH 6.6 or 7.2 with 4 N NaOH; 7 μg of EDDA per ml was added to limit the availability of iron in the medium.

[b] ^{125}I-labeled 30% saturated Fe-transferrin (Tf) or iron-free apotransferrin (ApoTf) was added to a final culture concentration of 2.5 mg/ml.

[c] $F_{(3,10)} = 0.96$. Variances within and between the means for transferrin and apotransferrin per cell are insignificant to the 95% confidence level.

[d] ND, Not determined.

binding sites were directly involved in the removal of iron from this molecule. Apotransferrin was bound to the same extent as 30% iron-saturated transferrin, suggesting that the meningococcus was incapable of distinguishing between loaded and iron-free transferrin at the concentrations used. Since iron-sufficient cells were capable of binding approximately the same number of transferrin molecules as iron-starved cells, the ability of the meningococci to bind transferrin appears to be independent of their capacity to incorporate iron from it.

No accumulation of iron from transferrin occurred when the meningococcal respiratory chain was blocked by cyanide, azide, or antimycin A (Table 2). Furthermore, cells exposed to trypsin or a temperature of 60°C for 5 min were also rendered incapable of removing iron from transferrin. Although iron uptake was dependent on a functional respiratory chain, the amount of transferrin bound to the surface of cyanide-treated cells was similar to that for metabolically active cells (Table 1).

Previous results from our laboratory (6) and those we present here indicate a cell surface mechanism for the removal of iron from transferrin. However, in view of the recent report of Yancey and Finkelstein (23) it was necessary to consider alternative explanations. If the meningococcus secreted (cell-free) low-molecular-weight siderophores, the mobilization of the iron from transferrin would occur as an event independent of the cells, and the subsequent incorporation of iron into the cell would presumably involve a siderophore-cell interaction rather than a direct transferrin-cell interaction. To test directly the possibility of siderophore involvement, ^{59}Fe-transferrin was immobilized by linking it to cyanogen bromide-activated Sepharose. The complex was then suspended in the cell-free NDM in which the iron-limited cells had been incubated for 60 min. The cell-free supernatant fluids were completely ineffective in mobilizing ^{59}Fe from the transferrin-Sepharose preparation as evidenced by the release of <0.1% of the radioactive label. However, fresh NDM supplemented with 100 mM citrate or pyrophosphate, two relatively weak iron chelators, released 60- or 120-fold more iron, respectively, indicating that the iron within the Sepharose was not totally unavailable to soluble factors in the medium. Although such evidence taken alone has its limitation, when considered with other evidence it takes on greater significance. For example, iron-starved meningococci were unable to obtain the required iron for growth, even after a 6-h incubation, when bacteria were separated from Fe-transferrin by a membrane with a 12,000-dalton exclusion limit (6).

Microbial siderophores have a high specific affinity for iron. Therefore, if the iron uptake from transferrin were dependent upon an extracellular or cell surface-associated siderophore, one could expect the rate of iron uptake to be

TABLE 2. Effect of heat treatment, trypsin treatment, and exposure to respiratory inhibitors on the uptake of iron from human transferrin by *N. meningitidis*

Treatment[a]	pmol of Fe/10^9 cells	% Uptake
Control	650	100
Cyanide (500 μM)[b]	10	1.5
Antimycin A (100 μM)[b]	27	4.1
Azide (5 mM)[b]	26	4.0
60°C, 5 min	16	2.5
Trypsin (100 μg/ml), 15 min	14	2.1

[a] *N. meningitidis* was cultured in MH medium containing 7 μg of EDDA (pH 7.2) per ml and suspended in NDM with no added iron (pH 7.4) before the addition of 30% saturated ^{59}Fe-labeled transferrin.

[b] Respiratory inhibitors were added to cultures 5 min before the addition of ^{59}Fe-labeled transferrin.

independent of the apotransferrin concentration in the environment, i.e., a siderophore should exhibit recognition and high affinity for the iron only. To test further the possibility of siderophore-mediated iron uptake, 2.5 mg of 30% saturated transferrin per ml (the concentration in normal serum) was supplemented with a 20-fold excess concentration of apotransferrin. Under these conditions, the rate of iron uptake decreased dramatically (>90%), indicating that, indeed, the transferrin molecule and not the iron was recognized at the cell surface (Fig. 4). Bovine serum albumin, a protein of similar molecular weight, was found to have no effect whatever on iron uptake when added in 20-fold molar excess over the 30% saturated transferrin. Therefore, the inhibition in the presence of apotransferrin was not merely due to interference by the greatly increased protein concentration in the cell environment. Such results are inconsistent with the concept of a soluble cell-free siderophore.

In view of the evidence that specific recognition of the transferrin molecule is essential for removal and uptake of iron, the initial rate of iron incorporation into the cell can be taken as a direct reflection of the turnover of transferrin at sites on the cell surface. Since a 30% saturated transferrin population contains a large proportion of apotransferrin molecules, which we have shown compete directly with transferrin, saturation kinetics experiments were also carried out with 100% saturated transferrin. The initial rate of iron uptake (Fig. 5) was limited at transferrin concentrations below 30 μM, irrespective of the level of transferrin iron saturation. The estimated dissociation constant for transferrin at the cell surface that can be determined from these data is 9.4×10^{-6}. The lower rates of uptake observed at higher concentrations of 30% saturated transferrin would be expected if the dissociation constant for apotransferrin were higher than that of transferrin. The means by which iron is removed from the transferrin molecule and the mechanisms for translocation of the iron into the cells remain unknown.

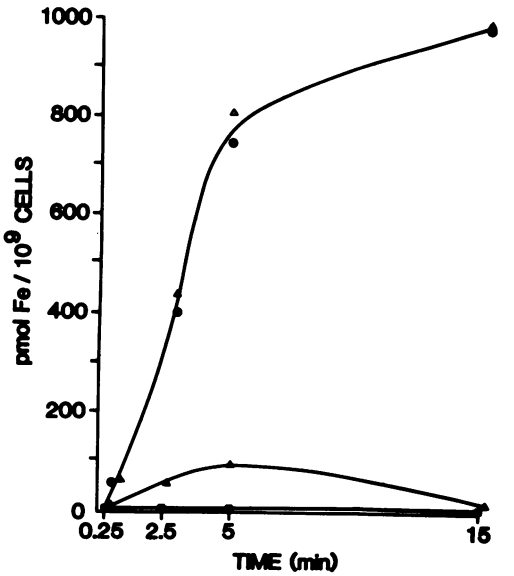

FIG. 4. Uptake of iron from ^{59}Fe-transferrin by *N. meningitidis* cultured in MH-EDDA medium at pH 7.2. Cells were suspended in iron-free NDM (pH 7.4) containing no additions (●) or 50 mg of BSA (△), ApoTf (▲), or 30% saturated ^{56}Fe-transferrin (■) per ml 2 min before the addition of ^{59}Fe-transferrin.

DISCUSSION

During in vitro experiments a variety of host molecules can supply *N. meningitidis* with the iron essential for its continued growth (7). Within the host, however, iron is sequestered intracellularly or is associated with high-affinity iron-binding proteins which function in the transport and storage of the metal. One such protein, the serum glycoprotein transferrin, is an important potential source of iron during the development of meningococcemia. The iron on transferrin is very tightly bound ($K_a = 10^{36}$) (1) and, consequently, is unavailable to many microorganisms. In contrast, meningococci are able to grow using transferrin-bound iron as their sole iron source (6). Holbein (13) has presented evidence for a central role of transferrin in support of meningococcal bacteremia in mice; meningococcemia could be sustained only as long as iron levels on transferrin were maintained.

Many microbes obtain iron from otherwise inaccessible sources by excreting high-affinity iron chelators, siderophores. In a report by Kvach et al. (14), enterobactin formed a complex with transferrin to provide virulent *Salmonella typhimurium* with iron. Lipopolysaccharide-defective strains were unable to adsorb the transferrin-iron-enterobactin complex and, therefore, failed to utilize transferrin-bound iron. These authors concluded that the ability to bind the complex was necessary for the microbe to initiate infection, whereas siderophore production permitted continued growth. Iron-binding catechols produced by *Escherichia coli* were able to overcome serum bacteriostasis by removing transferrin-bound iron and transporting the iron to the cell (19).

Very recently, Yancey and Finkelstein reported small amounts of hydroxamate-type molecules with iron-chelating ability in supernatant fractions of pathogenic neisserial cultures (23). With reference to a report of Archibald and DeVoe (6), they raise the possibility that chelating molecules associated with the meningococ-

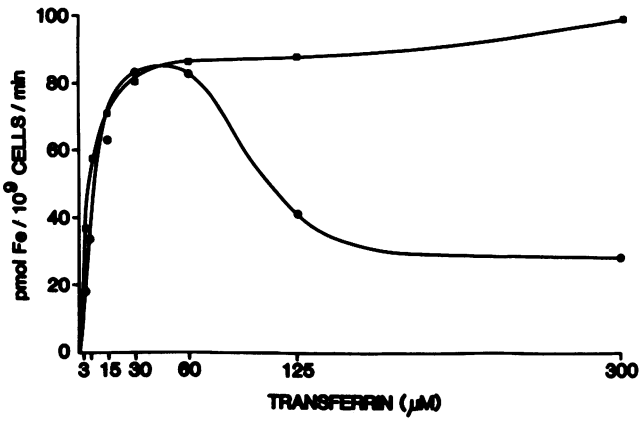

FIG. 5. Initial rates of iron uptake over first 5 min from various concentrations of 30% (●) or 100% (■) saturated ^{59}Fe-transferrin.

cal surface are responsible for the uptake of iron from transferrin. Since such siderophores are highly specific for Fe^{3+}, their function would presumably remain unaffected by other molecules in the environment. We have shown that the meningococcal acquisition of iron from transferrin is impeded by the presence of excess apotransferrin, but not by bovine serum albumin. Although it is not known whether the calculated number of surface receptors are all functional with respect to the unloading of transferrin, our evidence indicates that the recognition of the protein on the cell surface is essential for the subsequent uptake of iron. In view of this evidence, the mechanism proposed by Yancey and Finkelstein (23) would be tenable only as a secondary event after the surface recognition of transferrin. There may be a role for a low-molecular-weight chelator in the meningococcus to transport iron within the cell after its incorporation from transferrin, but our results argue against a primary function for either a cell-free or surface-associated siderophore in the initial recognition of iron on the transferrin molecule. It is conceivable that the transferrin molecule is degraded enzymatically on the cell surface, which could cause a release of iron to the cell. This possibility cannot be ruled out by the experiments presented here.

Acquisition of iron from chelates or complexes in the absence of siderophores is not unique to the meningococcus. Perry and Brubaker (17) have reported that *Yersinia pestis* is able to obtain iron from hemin without the benefit of excreted siderophores. The mechanism we have described for the meningococcal acquisition of iron from transferrin may be analogous to the system operating in reticulocytes and cultured mammalian cell lines, i.e., the saturable, reversible binding of transferrin to a membrane receptor preceding incorporation of iron (12, 21). The temperature sensitivity of the iron uptake system reported here is not consistent with the heat-stable properties of low-molecular-weight hydroxamate chelators. The inability of trypsin-treated *N. meningitidis* to accumulate iron implicates the involvement of protein(s) on the cell surface in the uptake. In another report from this laboratory, Simonson et al. (20) showed that isolated meningococcal outer membranes were capable of the energy-independent removal of iron from its dicitrate complex. In contrast, the unloading of transferrin at the cell surface requires metabolic energy, although the binding of the Fe-transferrin complex to the outer membrane occurs in the presence of cyanide.

Evidence suggesting that iron uptake from transferrin contributes to the pathogenicity of the bacterium is strengthened by the finding that nonpathogenic neisseriae are unable to utilize transferrin-bound iron. Brener et al. (8), in a recent report from this laboratory, showed that growth of *N. meningitidis* under iron-limited conditions at low pH, conditions simulating an inflammatory reaction, produced a 1,150-fold increase in the relative virulence for the mouse. A variety of phenotypic changes were observed that accompanied this increased virulence, including alterations in the outer membrane protein profile. It is not surprising that a pathogen, when exposed to adverse environmental conditions, responds by altering the properties of the cell surface in an effort to adapt to the new conditions. The fact that cells cultured at pH 6.6 exhibited an increased capacity to accumulate iron from transferrin may be significant in light of the lowered pH during pharyngitis. In addition to the lowering of pH, the release of lactoferrin from neutrophil granules at the site of inflammation could well induce the phenotypic

changes associated with the greatly enhanced virulence, in particular the ability to accumulate iron from transferrin.

The ability of a pathogen to compete successfully for transferrin-bound iron has been considered a virulence factor, as this property would confer a distinct advantage in the establishment and maintenance of infection. As humans are the only natural reservoirs for the meningococcus, it comes as no surprise that sophisticated mechanisms, such as we have described, have evolved to facilitate the acquisition of this vital nutrient from this host.

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