Identification of an outer-membrane haemoglobin-binding protein in *Neisseria meningitidis*

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Although *Neisseria meningitidis* can use haemoglobin as an iron source *in vitro*, the mechanism of haemoglobin-iron uptake is unknown. Using a biotinylated human haemoglobin probe in a solid-phase dot-binding assay, haemoglobin-binding activity was detected in total membranes derived from meningococci grown under iron-limited but not iron-sufficient conditions. In competition binding experiments, bovine and human haemoglobin could abrogate binding. In contrast, no binding inhibition was seen with ferric nitrate, protoporphyrin IX, and iron-loaded human transferrin. The ability of both haemin and catalase, a nonhaemoglobin haem-containing compound, to inhibit binding competitively suggested that the ligand recognized by the binding protein is the haem moiety. Scatchard plot analysis revealed a heterogeneous receptor population. Limited proteolysis with proteinase K abolished binding activity, suggesting a haemoglobin-protein interaction. Detection of activity in a whole-cell binding assay demonstrated that this haemin-binding protein was surface exposed. In a limited survey of meningococcal strains, the presence of haemoglobin-binding activity in all isolates indicated that expression of this binding protein is not serogroup specific.

Introduction

Neisseria meningitidis, a Gram-negative diplococcus, is an obligate human pathogen (DeVoe, 1982). Meningococcal disease, whose principal clinical expression is meningitis, continues to exert significant worldwide morbidity and mortality (Greenwood, 1984; Peltola, 1983). In the United States, this organism is the second most common cause of bacterial meningitis (Harrison & Broome, 1987; Schlech *et al.*, 1985), with an annual incidence of one to three per 100 000 confined to small focal outbreaks or case clusters (Harrison & Broome, 1987; Schlech *et al.*, 1985). In contrast, epidemics of group A meningococcal meningitis occur at ten to twelve year intervals in the 'meningitis belt' of sub-Saharan Africa, with an attack rate of 1% of the population and with an estimated annual incidence of 70 per 100 000 (Greenwood, 1984; Peltola, 1983).

A plethora of epidemiological and experimental evidence supports the premise that the ability of a

bacterial pathogen to acquire iron is a critical determinant in pathogenesis (Bullen, 1981; Finklestein *et al.*, 1983; Weinberg, 1984). The meningococcus is no exception. One such example is the enhanced lethality of meningococci following the addition of iron-dextran (Holbein, 1980) or iron-loaded transferrin (Holbein, 1981; Schryvers & Gonzalez, 1989) in a murine infection model. Similar observations pertain to the growth-promoting effects of haemoglobin (Brodeur *et al.*, 1985).

The mechanism of iron acquisition in *N. meningitidis* is unique. Whereas most human bacterial pathogens utilize siderophores, low-molecular-mass compounds that chelate iron followed by binding to their cognate receptor and subsequent internalization (Crosa, 1989; Neilands, 1982), as exemplified by the enterobactin system in *Escherichia coli* (Crosa, 1989), the meningococcus produces no siderophores (Simonson *et al.*, 1982). Rather, this bacterium uses a siderophore-independent receptor-mediated pathway to acquire iron from lactoferrin (Archibald & DeVoe, 1979; Schryvers & Morris, 1988a) and transferrin (Schryvers & Morris, 1988b). A similar system is operative in *Neisseria gonorrhoeae* (Lee & Schryvers, 1988) and *Haemophilus influenzae* (Schryvers, 1989).

Although haem and haemoglobin can supply the requisite iron for growth of iron-limited meningococci in

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Abbreviations: BHI, brain-heart infusion; biotin-X-NHS, biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester; EDDA, ethylenediamine di-*o*-hydroxyphenylacetic acid; SA-HRP, streptavidin-horse-radish peroxidase conjugate.

vitro (Mickelsen & Sparling, 1981) and *in vivo* (Schryvers & Gonzalez, 1989; Brodeur *et al.*, 1985), nothing is known of the mechanism of haem-iron uptake. Several observations have implied that a separate receptor-mediated process is involved in the uptake of haem-iron. Firstly, a pleiotropic meningococcal iron-uptake mutant that has lost the ability to use either lactoferrin or transferrin as the sole iron substrate has retained the ability to use haem (Dyer *et al.*, 1988). Secondly, human haemoglobin is incapable of binding to either the meningococcal lactoferrin (Schryvers & Morris, 1988*a*) or transferrin receptor (Schryvers & Morris, 1988*b*). This evidence suggests that the haem-iron utilization pathway in *N. meningitidis* is distinct from that of lactoferrin and transferrin and, furthermore, suggests that a haem-binding protein exists in *N. meningitidis* that is different from either the lactoferrin or the transferrin receptor. Therefore, assuming that the initial event in haem-iron uptake proceeds via such an analogous receptor-mediated interaction, the objective of this study was to identify such a cell-surface component by developing a solid-phase dot-binding assay specific for this constituent.

Methods

Bacterial strains and growth conditions. *N. meningitidis* B16B6, a standard serotyping strain, is a serogroup B, serotype 2A strain kindly provided by A. Schryvers (University of Calgary, Calgary, AB, Canada). Clinical meningococcal isolates representing serogroups A, C, X, Y and W135 were obtained from Foothills Hospital, Calgary, Alberta, Canada. *Pseudomonas cepacia* 715j (McKevitt *et al.*, 1989) was supplied by D. E. Woods (University of Calgary, Calgary, AB, Canada). *Escherichia coli* Sure strain was obtained from Stratagene. Organisms were maintained as stock cultures in skimmed milk at -70°C and, prior to use, were grown on chocolate agar plates supplemented with 1% (v/v) CVA enrichment (Gibco) at 35°C in an atmosphere containing 5% (v/v) CO_2 .

Chemicals. Human and bovine haemoglobins, bovine catalase, equine cytochrome c_{III} , iron-loaded human transferrin, bovine haemin, ferric nitrate, protoporphyrin IX and EDDA (ethylenediamine di-*o*-hydroxyphenylacetic acid) were purchased from Sigma. All the iron- and haem-containing compounds were more than 95% pure according to the manufacturer. Desferrioxamine was obtained from Ciba-Geigy Canada. Proteinase K was obtained from Gibco BRL. Streptavidin-horseradish peroxidase (SA-HRP) conjugate was from Bio/Can Scientific. Biotin-X-NHS (biotinyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester) was from Calbiochem. With the exception of bovine haemin, which was dissolved in a small volume of 10 mM-NaOH, and protoporphyrin IX, which was solubilized in a small volume of 10 mM-acetic acid, all of the iron and haem-containing compounds were solubilized in deionized water immediately prior to use.

Biotinylation of human haemoglobin. Preparations of human haemoglobin were equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, by cycles of gel filtration and ultrafiltration and diluted to 1 mg ml^{-1} . Biotin-X-NHS (250 μg) dissolved in 16 μl dimethylformamide was added to each millilitre of the protein solution, and the mixture was incubated with gentle agitation at 4°C for 2 h. The reaction was terminated by the

addition of 100 ml glycine (10 mg ml^{-1}) to each 1 ml portion, and the mixture was incubated for a further 2 h at 4°C . To remove excess unbound biotin, the samples were dialysed against three changes of 50 mM-Tris/HCl pH 8.0, 100 mM-NaCl and one change of 50 mM-Tris/HCl pH 7.5. The dialysed samples were concentrated with an Amicon filter cone and stored at 4°C .

Membrane preparation. Crude total membranes were prepared as previously described (Lee & Schryvers, 1988) from cells grown in iron-replete (brain-heart infusion [BHI] broth [Difco] alone) and iron-limited BHI broth (rendered iron-deficient by the addition of an iron chelator, either EDDA or desferrioxamine, to a final concentration of $100\text{ }\mu\text{M}$) conditions. Fractionation of total membranes into outer and inner membrane components was achieved by selective detergent solubilization using Triton X-114 (Schnaitman, 1971).

Growth curves. To evaluate the ability of various haem-containing proteins to serve as the sole exogenous source of iron for *N. meningitidis* B16B6, a series of growth experiments was conducted. After 12 h of growth at 35°C in an atmosphere of 5% (v/v) CO_2 on chocolate agar, organisms were inoculated into BHI broth rendered iron-deficient by the addition of $100\text{ }\mu\text{M}$ -EDDA to a starting OD_{600} of 0.010, as measured using a Pye Unicam PU8800 spectrophotometer (Unicam Analytical). This culture was shaken at 35°C in an atmosphere of 5% CO_2 until mid-exponential phase growth was achieved (OD_{600} 0.12–0.14). Aliquots were removed to inoculate, to an initial OD_{600} of 0.010, fresh pre-warmed BHI broth containing $100\text{ }\mu\text{M}$ -EDDA to which were added the various haem sources at molar equivalent amounts of haem. Growth at 35°C in 5% CO_2 was monitored hourly. All experiments were performed in triplicate.

Haemoglobin-binding assay. Haemoglobin-binding activity was detected using a solid-phase dot-binding assay. For the whole-cell binding assay, 12–14 h old organisms, that had been previously streaked on to chocolate agar and incubated at 35°C in the presence of 5% CO_2 , were suspended in BHI broth, with and without $100\text{ }\mu\text{M}$ -EDDA, to an initial OD_{600} of 0.04, as measured using the Pye Unicam PU8800 spectrophotometer. These cultures were shaken at 35°C in the presence of 5% CO_2 or in an anaerobic chamber (model 800A; Anaerobe Systems) rendered microaerophilic (5% O_2 , 10% CO_2 , 85% N_2 , by vol.), until mid-exponential phase growth was achieved (OD_{600} 0.12–0.14). Aliquots were removed and used to inoculate fresh pre-warmed BHI broth with and without $100\text{ }\mu\text{M}$ -EDDA. Following this second growth cycle to ensure depletion of internal iron stores, aliquots were again removed at mid-exponential phase and the OD_{600} was adjusted to 0.5. After washing once with 50 mM-Tris/HCl, pH 7.5, 1 M-NaCl, 100 μl of each suspension (approximately 10^7 c.f.u.) was filtered on to nitrocellulose/cellulose acetate paper (Immobilon-NC, 0.45 micron HAHY paper, Millipore Canada) using a 96-well filter manifold (Minifold I, Schleicher and Schuell), and the paper was allowed to air dry. After blocking with 2% (v/v) Blotto (2% skimmed milk in TBS [50 mM-Tris/HCl, pH 7.5, 1 M-NaCl]) at 37°C for 2 h, the paper was washed three times with TBS. Biotinylated human haemoglobin was added in blocking solution at a concentration of 50 nM. The binding mixture was incubated at 37°C for 1 h. The blot was washed three times with TBS prior to the addition of SA-HRP conjugate at a concentration of 500 ng ml^{-1} in blocking solution. After a further incubation at 37°C for 1 h, followed by washing three times with TBS, the paper was developed with a chloronaphthol/hydrogen peroxide substrate mixture (HRP reagent, Bio-Rad) at room temperature for 5–20 min. The paper was washed with water to stop the reaction. Identical experiments were performed with total membranes, as described below. Specific binding was defined as the difference between biotinylated human haemoglobin bound in the presence and in the absence of $100\times$ concentration of nonbiotinylated human haemoglobin. Identical experiments were performed using $100\text{ }\mu\text{M}$ -desferrioxamine, in lieu of EDDA, as the iron chelator.

Competition binding assay. To assess the specificity of binding, competition binding assays with various iron- and haem-containing proteins were performed. Whole cells, which had undergone two cycles of iron-limited growth as described above, were applied to nitrocellulose paper. The paper was air dried and blocked in 2% Blotto at 37 °C for 2 h. Increasing concentrations of the various competing ligands were mixed with a fixed saturating concentration of biotinylated human haemoglobin (50 nM). The paper was secured in a microdot apparatus and the mixtures were loaded into separate wells at a volume of 200 µl. After incubating the apparatus at 37 °C for 1 h, any solutions remaining in the wells were removed by filtration. The paper was removed, washed three times with TBS, and developed as described above.

Total membrane binding assay. Suspensions (2 µl) of crude total membranes, adjusted to a final concentration of 1 mg ml⁻¹ in 50 mM-Tris/HCl, pH 7.5, or dilutions, were immobilized onto Immobilon-NC paper, permitted to air dry and then blocked with 2% Blotto for 2 h at 37 °C. The paper was probed with biotinylated human haemoglobin as described above. Competition assays were conducted using the protocol described for the whole-cell binding assay.

Response to iron limitation. Two approaches were used to determine whether the expression of haemoglobin-binding activity was iron regulated. First, *N. meningitidis* B16B6 was grown in broth cultures in which iron limitation was progressively imposed by the addition of increasing concentrations of EDDA (from 0 to 150 µM). Samples from these cultures at mid-exponential phase growth were examined for haemoglobin-binding activity. Second, Fe(NO₃)₃ (150 µM) and bovine haemin (12 µM) were added to iron-starved cultures of *N. meningitidis* B16B6 and served as the sole source of exogenous iron. Aliquots were removed at mid-exponential phase growth and examined for haemoglobin-binding activity as described above.

Quantification of haemoglobin-binding activity. This was done by scanning the blots with a Zeineh video laser densitometer model SLD 1D/2D set in reflectance mode interfaced with an IBM PC computer incorporating the 1D/2D Soft Laser Scanning software package (Biomed Instruments, Fullerton, CA, USA).

Assessment of whole-cell integrity. The extent of cell lysis of cells that were grown under iron-rich and iron-restricted conditions was assessed visually by phase-contrast microscopy using an Olympus model BH-2 microscope; and biochemically by the presence of NADH oxidase activity, a cytoplasmic membrane-bound enzyme (Osborne *et al.*, 1972). Sonicated whole-cell samples and the 50 mM-Tris/HCl buffer, pH 8.0, served as the positive and negative controls, respectively, for the NADH oxidase enzyme assay.

Proteolytic digestion of *N. meningitidis* whole cells. Aliquots (1 ml) of cells prepared for the whole-cell assay (see above) underwent timed proteolytic digestion at 37 °C with addition of proteinase K to a final concentration of 50 µg ml⁻¹. Proteolysis was terminated after 5, 10, and 30 min intervals with the addition of the serine protease inhibitor PMSF to a final concentration of 200 µg ml⁻¹. The resultant digests were subjected to the whole-cell binding assay.

Identification of the meningococcal transferrin-binding protein. Transferrin-binding protein activity was assessed by using a horseradish peroxidase probe in the previously described solid-phase dot enzyme assay (Schryvers & Morris, 1988a).

Protein concentration determination. Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard.

Electrophoresis. Biotinylated and nonbiotinylated human haemoglobin were analysed by nondenaturing PAGE using a modification of the discontinuous buffer system of Laemmli (1970), in which

2-mercaptoethanol and SDS were omitted from the sample and running buffers and the sample was not subjected to thermal denaturation before electrophoresis. Gels comprised 5% (w/v of acrylamide) stacking and 12% (w/v of acrylamide) separating components with 0.8% bisacrylamide. The resultant gels were stained with silver (Oakley *et al.*, 1980). Biotinylated and unlabelled human haemoglobin separated in duplicate gel portions were electroblotted on to nitrocellulose membranes by the method of Towbin *et al.* (1979). The membranes were subsequently probed with the SA-HRP conjugate and developed with chloronaphthol/hydrogen peroxide substrate.

Results

Biotinylated human haemoglobin

When subjected to nondenaturing PAGE and stained with silver, the biotinylated human haemoglobin migrated as a single band identical to that of the unlabelled authentic human haemoglobin standard (data not shown). When the corresponding electroblot was probed with the SA-HRP conjugate and developed with the chloronaphthol substrate, a single band was seen with the transferred biotinylated haemoglobin (Fig. 1, lane B). This band exhibited the same mobility as the unlabelled and biotinylated haemoglobins separated by PAGE and silver-stained (data not shown). The absence of a signal emanating from the electroblotted unlabelled human haemoglobin (Fig. 1, lane A) indicated that the band detected from the biotinylated haemoglobin (Fig. 1, lane B) was due to specific interaction of the SA-HRP conjugate with the biotin moiety of the transferred protein, and did not represent nonspecific binding of the conjugate to haemoglobin itself. These results provide evidence that no significant degradation of the ligand occurred during the biotinylation procedure.

Haemoglobin-binding assay

Preliminary experiments to determine the time course of association of the biotinylated human haemoglobin with iron-restricted *N. meningitidis* whole cells grown to mid-exponential phase indicated that equilibrium occurred at 1 h at 37 °C (data not shown). Binding at 4 °C or 25 °C produced identical results (data not shown). In view of the necessity for equilibrium binding for Scatchard analysis, all subsequent binding experiments were performed for 1 h at 37 °C. Haemoglobin-binding activity required the presence of both whole cells and the biotinylated ligand (Fig. 2, A). When cells were not applied to the nitrocellulose membrane, no binding activity was seen (Fig. 2, D). This result eliminated the possibility that nonspecific attachment of the probe to the Immobilon-NC paper contributed to the observed signal. Similarly, no signal was seen when the chloronaphthol substrate alone was added to immobilized

Fig. 1. Electroblot of biotinylated human haemoglobin. Five micrograms each of human haemoglobin (lane A) and biotinylated human haemoglobin (lane B) were separated in a 12% nondenaturing PAGE as described in Methods. The separated proteins were electroblotted on to a nitrocellulose membrane and probed with SA-HRP. The blot was developed by the addition of chloronaphthol substrate. The arrowhead indicates biotinylated protein in the stacking-separating gel interface that failed to enter into the separating gel.

Fig. 2. Biotinylated human haemoglobin solid-phase dot-binding assay. Aliquots (10^7 c.f.u.) of *N. meningitidis* B16B6 grown under iron-restrictive conditions were immobilized on to nitrocellulose membrane. Cells were probed with the biotinylated human haemoglobin followed by the SA-HRP conjugate and the chloronaphthol substrate, as described in Methods (A); chloronaphthol substrate alone (B); and SA-HRP conjugate followed by the chloronaphthol substrate (C). In sample D, the nitrocellulose membrane without applied cells was probed for binding activity using the dot-binding protocol.

whole cells (Fig. 2, B), indicating that intrinsic peroxidase activity present in the whole-cell membranes was not responsible for binding activity. The absence of a signal with the addition of the SA-HRP alone to the whole cells also excluded nonspecific binding of this conjugate to the whole cell preparations (Fig. 2, C). Identical results were obtained using iron-limited total membranes (data not shown).

Fig. 3(a) shows the effect of increasing amounts of iron-limited whole cells on biotinylated human haemoglobin ligand binding. Total human haemoglobin binding increased in proportion to the amount of whole cells immobilized on the nitrocellulose, whereas nonspecific binding was negligible (data not shown). In the reciprocal experiments when 10^7 c.f.u. of iron-starved whole cells were incubated with increasing amounts of biotinylated human haemoglobin, saturable binding was observed (Fig. 3b). Scatchard analysis (Scatchard, 1946) of the binding data revealed a curvilinear plot, indicating the presence of two populations of binding protein (Fig. 3c). A high-affinity receptor with an apparent dissociation constant, K_d , of 2.52×10^{-7} M co-existed with a low-affinity receptor (K_d 7.9×10^{-6} M). Because all the available unoccupied binding sites would not be exposed by using the dot-binding assay, as would presumably occur with the more traditional binding-assay methods of equilibrium and flow dialysis, these kinetic data represent a minimal estimate. Alternatively, the presence of a heterogeneous receptor population may be an artifact of the dot-binding assay, and this interpretation cannot be excluded.

Specificity of whole-cell binding for human haemoglobin

The specificity of whole-cell binding activity for human haemoglobin was addressed in a series of competition experiments. The binding of biotinylated human haemoglobin to meningococci was specifically blocked by competition with unlabelled bovine haemin and the haemoproteins human haemoglobin, bovine haemoglobin and bovine catalase in a concentration-dependent manner (Fig. 4a). In contrast, no binding inhibition was observed with another haem-containing protein, equine cytochrome c_{III} (Fig. 4b); or with protoporphyrin IX, the immediate precursor of haem in the haem biosynthetic pathway (data not shown). Iron-loaded human transferrin, a nonhaem iron-containing glycoprotein, and $\text{Fe}(\text{NO}_3)_3$ also failed to competitively inhibit binding of biotinylated human haemoglobin to meningococcal cells (Fig. 4b). Identical results were seen with iron-limited total membranes (data not shown).

Growth curves

The functional correlates of this binding specificity were determined by examining the ability of the various competing haem-containing ligands to support the growth of iron-restricted cultures of meningococci. A representative experiment is shown in Fig. 5. The results recapitulated those seen in competition assays. Bovine haemin, human and bovine haemoglobin, and bovine catalase could each serve as the sole exogenous iron

Fig. 3. Kinetics of the biotinylated human haemoglobin (b-hHb) solid-phase dot-binding assay. (a) Concentration response curve for number of bacteria per spot binding to biotinylated human haemoglobin. Aliquots of *N. meningitidis* B16B6 grown under iron-limited conditions were applied on to nitrocellulose filters, incubated with blocking buffer, and then probed for haemoglobin-binding activity with biotinylated human haemoglobin in the presence or absence of 100-fold excess of unlabelled human haemoglobin. After developing, the blots were quantified using a scanning densitometer. The amount of haemoglobin bound is expressed as the area under the peaks, in arbitrary units. Results depict specific binding. Specific binding was determined by subtracting nonspecific binding from total binding. (b) Saturability of biotinylated human haemoglobin binding. A fixed amount of meningococci grown under iron-restricted conditions (10^7 c.f.u.) was immobilized on to triplicate nitrocellulose membranes which were probed with increasing concentrations of biotinylated human haemoglobin (0.496–62.5 nM) in the presence or absence of 100-fold excess unlabelled human haemoglobin. After developing, specific binding activity was quantified as in (a). The results in (a) and (b) each represent the means of three experiments performed in duplicate. (c) Scatchard analysis of specific binding of biotinylated human haemoglobin to *N. meningitidis* B16B6. B, bound; F, free.

source for the growth of iron-limited meningococci. Biotinylated human haemoglobin retained the capacity to function as an iron substrate, indicating that its chemical modification did not significantly impair its legitimacy as an authentic ligand. In contrast, consistent with its inability to inhibit binding in the competition studies, cytochrome c_{III} could not support the growth of iron-restricted meningococci. The optical densities of all the cultures measured after 24 h of growth were identical to that seen after 5 h, indicating that stationary growth was achieved at 5 h (data not shown).

Assessment of whole-cell integrity

Cells grown under either iron-limited or iron-rich conditions and subsequently suspended in Tris buffer were intact when examined by phase-contrast microscopy (data not shown). In contrast, only fragmented cells and cell debris were seen in the sonicated samples (data not shown). Minimal NADH oxidase activity was detected in the supernatant of cells grown under either iron-limited or iron-rich conditions (data not shown). The whole-cell lysates demonstrated pronounced NADH oxidase activity (data not shown). Control mixtures comprising either the Tris buffer or supplemented BHI broth with and without $100 \mu\text{M}$ -EDDA were devoid of NADH oxidase activity. These results indicated that whole-cell integrity was maintained in the cultures used

in the whole-cell binding assay. The minimal cell lysis present excluded significant contamination of the whole-cell aliquots with intracellular sequestered haem-containing compounds, such as cytochrome proteins.

Cellular localization of the meningococcal haemoglobin-binding protein

Haemoglobin-binding activity was present in Triton-insoluble membrane extracts derived from crude total membranes prepared from cells grown under iron-deficient conditions (Fig. 6a). In contrast, no haemoglobin-binding activity was observed in the Triton-soluble membrane fraction (Fig. 6a). Because membrane partitioning is preferential by detergent solubilization and not absolute, this result implied that the cell component possessing the haemoglobin-binding property resided in the outer membrane. The parallel whole-cell binding studies that were conducted corroborated its outer membrane topography. The expression of haemoglobin-binding activity from whole cells grown under iron-limited conditions (Fig. 6b), and not from whole cells grown under iron-rich conditions (Fig. 6b), indicated that this haemoglobin-binding moiety was both surface exposed and iron regulated. No signal was present when iron-limited supplemented BHI medium alone was subjected to the solid-phase dot-binding protocol (data not shown).

Fig. 4. Specificity of meningococcal haemoglobin-binding activity. (a) Effects of different haemoproteins on biotinylated human haemoglobin binding. Dot-blots of iron-limited meningococci (10^7 c.f.u.) prepared as described in the text were probed in competitive assays with biotinylated human haemoglobin (50 nM) in the presence of increasing concentrations of human haemoglobin (●), bovine haemoglobin (■), bovine haemin (◆), and bovine catalase (▲). Maximum binding is defined as the amount of biotinylated human haemoglobin associated with *N. meningitidis* B16B6 in the absence of competing ligands. Data represent the mean of three experiments performed in duplicate. (b) Effects of equine cytochrome c_{III} (○), $\text{Fe}(\text{NO}_3)_3$ (□), and 100% iron-loaded human transferrin (△). Competition binding experiments were performed as described in the text. Maximum binding is defined as the amount of biotinylated human haemoglobin retained on the nitrocellulose membranes in the absence of competing ligands. Data represent the mean of three experiments performed in duplicate.

Iron-regulated expression of the haemoglobin-binding protein

As noted above, haemoglobin-binding activity was only observed in intact whole cells grown in iron-limited conditions (Fig. 6b) and this activity increased with progressive iron restriction (Fig. 6b). Expression was independent of the iron chelator used to impose iron limitation (Fig. 6b). No binding activity was detected in cells grown under iron-rich conditions (Fig. 6b). Similarly, when iron-limitation was reversed by the addition of $150 \mu\text{M}$ - $\text{Fe}(\text{NO}_3)_3$, or $12 \mu\text{M}$ -haemin, binding activity was absent (Fig. 6c). These results indicated that haemoglobin-binding activity is iron-repressible. Examination of the crude total membranes that were derived from these cell cultures produced identical results (data not shown). Binding activity was absent in iron-rich

Fig. 5. Growth response of *N. meningitidis* B16B6 in iron-limited BHI broth without additional supplements (○), or with the addition of molar equivalents of haem (12 μM) as bovine haemin (■), human haemoglobin (●), bovine haemoglobin (◆), biotinylated human haemoglobin (▲), bovine catalase (□), and equine cytochrome c_{III} (△).

cultures of meningococci grown under microaerophilic conditions or at 30°C , indicating that neither oxidative stress nor reduced growth rate governed expression (data not shown).

Proteolytic digestion of meningococcal whole cells

Pre-incubation of iron-restricted whole cells with proteinase K abolished receptor activity in a time- and dose-dependent fashion (data not shown), suggesting that either a protein determinant(s) is involved in the interaction of the receptor with its ligand or that preservation of the surrounding cellular protein architecture is necessary for binding.

Presence of haemoglobin-binding activity in other meningococcal strains

In a limited survey of meningococcal isolates, haemoglobin binding activity was discerned in representative strains belonging to serogroups A, C, X, Y and W135 (Fig. 7). This result indicates a functional conservation of the haemoglobin-binding protein among clinically significant serogroups. Under similar iron-limiting conditions, no haemoglobin-binding protein expression was detected in *Pseudomonas cepacia* or *E. coli*.

Meningococcal transferrin-binding protein

Transferrin-binding protein activity was present only under conditions of iron-limitation (data not shown).

Fig. 6. (a) Outer-membrane location of haemoglobin-binding activity. Crude total membranes from *N. meningitidis* B16B6 grown under iron-restricted conditions were solubilized with Triton X-114. Aliquots of the Triton-insoluble and the Triton-soluble fraction (each 3 ng) were spotted on to nitrocellulose paper and probed for haemoglobin-binding activity as described in Methods. (b) Expression of haemoglobin-binding activity. Cultures of *N. meningitidis* B16B6 were grown in the presence of increasing concentrations of EDDA which produced increasing iron restriction. Aliquots from these cultures were examined for haemoglobin-binding activity as described in Methods. The bottom spot represents an aliquot of meningococci from a culture grown in the presence of the iron chelator desferrioxamine (DF). (c) Iron regulation of haemoglobin-binding activity. Iron-starved cultures of *N. meningitidis* B16B6 were provided with either Fe(NO₃)₃ or bovine haemin as the sole exogenous iron source at the indicated concentrations. Aliquots were removed at the mid-exponential phase of growth and examined for haemoglobin-binding activity using the solid-phase dot-binding assay as described in Methods.

Fig. 7. Haemoglobin-binding activity in other bacteria. Clinical isolates of *N. meningitidis* representing serogroups A, B, C, X, Y and W135, and *P. cepacia* (*P. cep.*) and *E. coli* Sure strain were grown under iron-limited conditions. Samples were removed at the mid-exponential phase of growth, immobilized on to nitrocellulose membrane, and probed for haemoglobin-binding activity as described in Methods.

Time course experiments showed that transferrin-binding protein expression occurred concurrently with haemoglobin-binding activity (data not shown). These results internally validated the protocols used for iron starvation and suggested that the expression of both binding proteins is coordinately regulated.

Discussion

Using a biotinylated human haemoglobin probe, an iron-regulated surface-exposed haemoglobin-binding protein has been identified in *N. meningitidis*. The binding kinetics of this protein for haemoglobin exhibit saturability, specificity and reversibility, characteristics satisfying the generally accepted functional definition of a receptor-ligand interaction (Kahn, 1976).

The binding kinetics of the meningococcal haemoglobin-binding protein are compatible with either a cooperative model, in which binding at one site modulates the affinity of the second binding site (Freifelder, 1982), or with a heterogeneous model, in which two independent binding sites compete for haemoglobin binding (Freifelder, 1982). Such receptor-ligand interactions are common in biological systems (Freifelder, 1982; Gammeltoft, 1984). For instance, a two-component iron-uptake mechanism has been proposed for the lactoferrin receptor in *Trichomonas vaginalis* (Lehker & Alderete, 1992).

The meningococcus exhibits a preferential ability to appropriate iron from transferrin relative to haem and haemoglobin (Schryvers & Gonzalez, 1989). Such a model might account for this functional hierarchy, despite the low K_d (0.7 μM) of the transferrin receptor (Tsai *et al.*, 1988). Assuming the presence of two binding sites with two distinct affinities for haemoglobin, binding of haemoglobin to the low-affinity component of the meningococcal haemoglobin-binding protein (K_d 7.9 μM), coupled subsequently with haem-iron translocation across the periplasmic space to the cytosol, may represent the rate limiting step(s) in meningococcal haemoglobin-iron uptake.

Several reasons may account for the inability of cytochrome c_{III} to supply the requisite haem-iron for the growth of iron-starved *N. meningitidis* B16B6. One explanation derives from the observation that cytochrome c_{III} was incapable of displacing haemin binding in the competition assays. This result implies that this haemoprotein is unable to bind to the haemoglobin-binding protein. Another explanation may be related to the ease of extraction of the haem prosthetic group. Unlike haemoglobin, in which haem release occurs readily under nondenaturing conditions (Smith *et al.*, 1991), haem dissociation in cytochrome c_{III} would be more difficult owing to the covalent attachment of haem to the polypeptide backbone (Dickerson *et al.*, 1971).

Although the possibility exists that the haemoglobin-binding protein represents a class of cytochrome proteins, this explanation is very unlikely for the following reasons. Most described cytochromes are periplasmic or cytoplasmic membrane-associated moieties (Meyer & Pistorius, 1989). In contrast, the haemoglobin-binding protein is a surface-exposed outer-membrane protein. Also inconsistent is the lack of enhanced production of the haemoglobin-binding protein under conditions of oxygen stress, a feature of most cytochromes (Gennis, 1987). These properties provide a cogent argument that this haemoglobin-binding protein is not a cytochrome.

The source of haemoglobin for *N. meningitidis* *in vivo* is unknown. Microangiopathic haemolytic anaemia is a hallmark of invasive meningococcal infection (DeVoe, 1982). This pathological process would liberate intracellular sequestered haemoproteins, such as haemoglobin, permitting their access by meningococci. Although free intravascular haemoglobin is avidly bound to its serum carrier protein haptoglobin, meningococci can successfully scavenge haem from this complex (Dyer *et al.*, 1987).

Because of their propensity to aggregate in aqueous solutions at physiological pH (Brown *et al.*, 1976; Muller-Eberhard & Nikkila, 1989), and because of their potential cellular toxicity due to participation in lipid peroxidative catalysis (Vincent, 1989), haem and other tetrapyrroles are always bound to protein (Muller-Eberhard & Nikkila, 1989). In man, a plethora of intracellular and extracellular haem-binding proteins have been characterized (Muller-Eberhard & Nikkila, 1989), some coupling transport with antioxidant functions (Gutteridge & Smith, 1988). Indeed, haem translocation across eukaryotic cell membranes is postulated to be a function of a haem-specific binding receptor (Galbraith *et al.*, 1985; Galbraith & McElrath, 1988; Grasbeck *et al.*, 1979; Smith & Morgan, 1984), whose expression is induced under iron limitation (Smith & Ledford, 1988).

The biochemical constraints imposed by free haem

prompt the speculation that an analogous haem-uptake mechanism exists in *N. meningitidis*, whereby the initial step in haemoglobin-iron uptake involves the interaction with a surface-accessible haemoglobin specific receptor. The iron-regulated outer-membrane haemoglobin-binding protein described in this report would be an attractive functional candidate. Indeed, surface-accessible haemin-specific binding proteins that bind haemoglobin have been recently isolated from two other human-specific bacterial pathogens, *N. gonorrhoeae* (Lee, 1992b) and *H. influenzae* (Lee, 1992a).

Evidence derived from the competition binding assays suggests that the haem moiety may be the ligand recognized by the meningococcal haemoglobin-binding protein. Both catalase, and significantly, haem alone are capable of abrogating binding in the competitive assays. The ability of catalase, a nonhaemoglobin haemoprotein, to inhibit binding suggests that the globin subunits of haemoglobin do not interact significantly with the binding protein. Competition binding experiments using isolated globin chains as the competing ligand may address this issue. However, the marked conformational differences between globin, and globin bound to its haem prosthetic group (Yip *et al.*, 1972; Waks *et al.*, 1973; Leutinger & Beychok, 1981) make this approach problematical. Haemin exhibited a diminished capacity, relative to the three other haemoproteins, human and bovine haemoglobin, and bovine catalase, to inhibit binding of the biotinylated haemoglobin probe competitively (Fig. 4a). This observation may reflect the tendency of haemin to aggregate in aqueous solutions (Brown *et al.*, 1976; Muller-Eberhard & Nikkila, 1989), a characteristic that would reduce the effective concentration of haemin available to compete for binding. In addition, this aggregating property offers a putative explanation for the enhanced ability of haemin to serve as an iron substrate (Fig. 5), despite being less effective at competition (Fig. 4a). The stacking of haemin molecules may permit several haemin moieties to bind to a single receptor, thereby effectively augmenting the ability of haemin to function as an iron source.

Furthermore, the inability of either iron or protoporphyrin IX to inhibit binding competitively suggests that the presence of iron within the tetrapyrrole ring is required for its recognition as a legitimate ligand. Alternatively, the binding protein may display a significant stereospecificity for haemin (iron protoporphyrin IX) relative to other haem analogues, such as protoporphyrin IX. Such a pronounced bias for the preservation of the central iron in the porphyrin ring is mirrored in the increased affinity of eukaryotic plasma membrane haem receptors for iron-containing metalloporphyrins (Galbraith *et al.*, 1985; Galbraith & McElrath, 1988). In this context, the effectiveness of

metalloporphyrins with substituted central metals in displacing haemoglobin binding would be of interest.

In conclusion, this study has identified the presence of an iron- and haemin-regulated surface-exposed haemoglobin-binding protein in *N. meningitidis*. Studies are under way to isolate the receptor polypeptide(s) and to delineate its role in haem-iron acquisition.

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