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A Monoclonal Antibody Directed against the 97-Kilodalton Gonococcal Hemin-Binding Protein Inhibits Hemin Utilization by *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae **expresses two hemin-binding proteins (HmBPs) of 97,000 and 44,000 in molecular weight. A murine monoclonal antibody (MAb) produced against the 97-kDa HmBP from *N. gonorrhoeae* PID543 specifically inhibited in a concentration-dependent manner the ability of hemin to promote growth. The anti-97-kDa HmBP MAb competitively inhibited binding of the 97-kDa HmBP to a hemin-agarose affinity column. In Western immunoblots, the MAb recognized the 97-kDa homologs from a limited survey of clinical gonococcal isolates. These results support the contention that the 97-kDa HmBP is involved in the gonococcal hemin acquisition pathway.**

Neisseria gonorrhoeae remains an important human bacterial pathogen. Gonococcal infections continue to exact a significant public health toll and economic burden (3, 4). The brunt of the morbidity resulting from gonococcal disease is borne by women of childbearing age, in the form of adverse reproductive outcomes such as ectopic pregnancy and involuntary tubal sterility (3, 4).

Despite the introduction of potent antimicrobial agents, the gonococcus has maintained its ecological niche in the human host (5). The mechanisms whereby the organism acquires iron exemplify this exquisite adaptation. In response to the iron-restricted environment encountered in the human genital tract, the gonococcus expresses a diverse array of surface-exposed proteins that exhibit a pronounced binding specificity for host iron-containing proteins. The interaction of these iron-containing proteins with this panoply of gonococcal receptors is considered to represent the initial step in the acquisition of iron from transferrin (17, 23), lactoferrin (17, 23), and hemin (14). Genetic investigations have corroborated such a role for the gonococcal transferrin-binding proteins (1, 2, 6, 7).

The mechanism of heme-iron uptake in *N. gonorrhoeae* is not well delineated. Two hemin-binding proteins (HmBPs) of 97 and 44 kDa that specifically recognize hemin and that are expressed in response to iron starvation have been isolated from the gonococcus (14). However, evidence showing their direct participation in the heme-iron acquisition pathway has been circumstantial. Therefore, the purpose of this investigation was to more precisely define the role of the 97-kDa HmBP in the gonococcal heme-iron uptake pathway by using, as a functional probe, a murine monoclonal antibody (MAb) raised specifically against the 97-kDa HmBP.

The five clinical isolates of *N. gonorrhoeae* used in this study were obtained from patients with pelvic inflammatory disease (PID543 [kindly provided by R. Brunham, University of Manitoba, Winnipeg, Manitoba, Canada] and PID5), uncomplicated cervicitis (C439), and uncomplicated urethritis (W159 and U750). Strains were maintained as stock cultures in 10% (wt/vol) skimmed milk and 10% (vol/vol) glycerol at −70°C. Prior to use, organisms were streaked onto chocolate agar and incubated at 35°C in an atmosphere of 5% CO_2 for 12 to 14 h. Batch cultures were inoculated with gonococci grown overnight on chocolate agar and were incubated at 35°C in an atmosphere of 5% CO_2 on an incubator-shaker, model G27 (New Brunswick Scientific Co., New Brunswick, N.J.). Each 2-liter flask contained 1.5 liters of SJ-GC medium (25) that was rendered iron deficient with the addition of the iron chelator desferrioxamine (Ciba-Geigy Canada Ltd., Mississauga, Ontario, Canada) to a final concentration of 100 μM. Cells were harvested at the mid-logarithmic phase of growth.

Crude bacterial total membranes were prepared as previously described (17). Protein concentrations were determined by the method of Lowry et al. (20) with bovine serum albumin as a standard.

HmBPs were isolated by ligand affinity chromatography by a batch affinity method as previously described (14) with modifications. Crude total membrane, adjusted to a final concentration of 0.25 mg/ml in 50 mM Tris-HCl–1 M NaCl (pH 8.0), was incubated with 95 nmol of hemin-agarose (30) (Sigma, St. Louis, Mo.) at 20°C for 1 h. The mixture was then centrifuged at 750 × *g* for 5 min, and the supernatant was discarded. The pelleted affinity gel was resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0), and the membrane was subsequently solubilized with the addition of sodium-EDTA (pH 8.0) and Sarkosyl NL30 to final concentrations of 10 mM and 0.75% (vol/vol), respectively. After incubation at 20°C for 1 h, the affinity resin was pelleted by centrifugation, then washed twice for 30 min each with high-salt buffer (50 mM Tris-HCl, 1 M NaCl, pH 8.0) containing 5 mM sodium-EDTA and 0.5% (vol/vol) Sarkosyl NL30, and finally washed once for 10 min with 50 mM Tris-HCl–100 mM NaCl (pH 8.0). Bound proteins were eluted by incubating the affinity resin at 20°C for 30 min with 50 μl of solution containing 500 mM Tris-HCl (pH 6.8), 2% (wt/vol) sodium dodecyl sulfate (SDS), 30% (vol/vol) glycerol, and 1% (vol/vol) 2-mercaptoethanol. A 25-μl aliquot was applied to an SDS–10% polyacrylamide gel for analysis by SDS-polyacrylamide gel electrophoresis (PAGE).

To produce the anti-97-kDa HmBP MAb, the 97-kDa HmBP from gonococcal strain PID543 was affinity isolated by ligand affinity chromatography and the HmBP was electroeluted from preparative SDS-polyacrylamide gels with a unidirectional electroelutor (model UEA; IBI, New Haven,

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Conn.). To remove the detergent, samples were dialyzed against three changes of 50 mM Tris-HCl (pH 8.0). The dialyzed HmBP was concentrated with a Minicon Macrosolute concentrator (Amicon, Danvers, Mass.) and then stored at −70°C. Female BALB/c mice (6 to 7 weeks old) (Simonsen Laboratories, Inc., Gilroy, Calif.) were immunized intravenously and intraperitoneally with a suspension of 20 μg of antigen and 50 μg of adjuvant peptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) (Sigma). Four intravenous booster injections of 20 μg each of the antigen were administered at 7- to 10-day intervals. Preimmune and immune sera were tested for reactivity to the 97-kDa HmBP by immunodot and Western blotting. Spleen cells were fused with NS1 myeloma cells, according to the protocol described by Longenecker et al. (19).

Antibodies secreted by the hybridomas were screened for binding to the gonococcal 97-kDa HmBP by a previously described enzyme-linked immunosorbent assay (ELISA) (21). Briefly, 200 μl of affinity-purified 97-kDa HmBP in carbonate buffer (pH 9.6) at a concentration of 1 μg/ml was used to coat the wells of microtiter plates. After a 12-h incubation at 4°C, the wells were washed three times with phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% (wt/vol) Tween 80 (PBS-Tween). Culture supernatants from the hybridomas were added, and the plates were incubated at room temperature for 2 h. Wells were washed three times with PBS-Tween, and a 1:2,000 dilution of peroxidase-conjugated rabbit anti-mouse antibody was added. The color reaction was obtained by the addition of hydrogen peroxide and ABTS (2,2′-azino-di-[3-ethyl-benzthiazoline]) as recommended by the supplier (Kirkegaard and Perry Laboratories). The A_{405} was read with an Autoreader (model EL310; Bio-Tek Instruments, Inc., Burlington, Vt.). Hybridomas that produced culture supernatants reacting with the 97-kDa HmBP were cloned by the limiting-dilution method. MAb isotype was determined by ELISA, with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin M (IgM) antiserum. Hybridomas were expanded and cultured in the peritoneal cavities of Pristane-primed BALB/c mice to obtain ascites fluid (19).

The anti-97-kDa HmBP MAb and a MAb against the meningococcal lactoferrin-binding protein (42-9-6-5) (a gift from A. Schryvers) were purified from mouse ascites fluid by affinity chromatography on a mannan-binding protein-agarose column (Pierce Chemical Co., Professional Diagnostic Bioscience, Inc., Aurora, Ontario, Canada) according to the protocol of the manufacturer. Fractions exhibiting an A_{280} greater than 0.02 were pooled, dialyzed at 4°C against several changes of 50 mM Tris-HCl (pH 8.0), and concentrated with an Amicon Macrosolute concentrator. Purity of the MAb preparation was ascertained by SDS-PAGE. The functional specificity of the purified MAb was determined by Western immunoblot analysis.

To examine whether the MAb was functionally capable of inhibiting heme and hemoglobin use, a series of growth experiments, in which either heme or hemoglobin served as the sole exogenous source of iron for *N. gonorrhoeae* PID543, were performed. After 12 h of growth on chocolate agar plates at 35°C in an atmosphere of 5% CO_2, organisms were inoculated into SJ-GC broth, made iron deficient with the addition of 150 μM desferrioxamine, to a starting A_{600} of 0.05, as measured with a Pye Unicam PU8800 spectrophotometer. This culture was shaken at 35°C in an atmosphere of 5% CO_2 until mid-logarithmic-phase growth was achieved. Aliquots were removed, to inoculate 6 ml of prewarmed iron-deficient SJ-GC broth containing 150 μM desferrioxamine and the anti-97-kDa HmBP MAb to an initial A_{600} of 0.05. Control samples contained 150 μM desferrioxamine and either the irrelevant IgM MAb directed against the meningococcal lactoferrin-binding protein or the dilution buffer. After incubation for 30 min at 35°C, bovine hemin, human hemoglobin, or iron-loaded human lactoferrin (purchased from Sigma) at molar equivalent amounts of iron was added to the cultures. To determine if either MAb exhibited a bactericidal effect, cell counts were performed on aliquots removed prior to and after the 30-min incubation. Growth at 35°C in 5% CO_2 was monitored by performing cell counts on aliquots removed at hourly intervals. All experiments were performed in triplicate.

The ability of the anti-97-kDa HmBP MAb to inhibit binding of the 97-kDa HmBP to hemin was assessed by conducting competition binding experiments in which total membranes derived from gonococcal cultures grown under iron-limited conditions were preincubated in 50 mM Tris-HCl–1 M NaCl (pH 8.0) at 35°C for 1 h with increasing concentrations of the MAb. The solutions containing the MAb and the total membranes were subsequently subjected to the hemin-affinity chromatography protocol, and the HmBPs were eluted from the hemin-affinity resin as described above. Because total membrane preparations would be expected to be enriched for the presence of the HmBPs compared with the equivalent amount of whole-cell protein, the concentration of MAb used in the competitive binding experiments was accordingly increased. Serial twofold dilutions of total membranes and whole cells were probed in immunodot assays with the anti-97-kDa HmBP MAb. The immunodots were scanned by laser densitometry in order to determine the whole-cell protein and the total membrane concentrations producing an equivalent signal. These concentrations were expressed as a ratio, and this result was used to calculate the concentration of MAb to be added in the competitive binding assays.

For the Western immunoblots, total membrane prepared from cells grown under iron-limited conditions (5 μg) and affinity-purified HmBPs (25 μl) were separated on an SDS–10% polyacrylamide gel and were electroblotted at 10 V of constant voltage for 12 h at 4°C onto a polyvinylidene difluoride membrane (Immobilon-P, 0.45-μm pore size; Millipore Canada) in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol, pH 8.3, by the method of Towbin et al. (29) with a Bio-Rad MiniTransblot apparatus. For the immunodots, 1-μg aliquots (1 mg/ml) of total membranes derived from cells grown under iron-limited conditions or affinity-purified 97-kDa HmBP from gonococcal strain PID543 were immobilized onto nitrocellulose-cellulose acetate paper (Immobilon-NC, 0.45-μm pore size; HAHY paper; Millipore Canada). After being blocked with 0.5% (wt/vol) skimmed milk in 50 mM Tris-HCl (pH 7.5)–1 M NaCl (TBS-M) for 1 h at 35°C, the membrane was reacted for 1 h at 35°C with a 1:5 dilution of the affinity-purified murine IgM MAb directed against the 97-kDa gonococcal HmBP. The immunoblot was then rinsed with three 5-min washes of TBS-M. A 1:2,500 dilution of the second antibody (goat antimouse IgM-HRP conjugate [Pierce Chemical Co.]) was allowed to bind for 1 h at 35°C, and this was followed by three 5-min washes with TBS-M. The blots were developed with a chloronaphthol-hydrogen peroxide substrate mixture (HRP reagent; Bio-Rad) for 10 min. The paper was washed with water to stop the reaction.

HmBPs were separated by SDS-PAGE with the discontinuous buffer system of Laemmli (13) with a Bio-Rad MiniProtean II apparatus as previously described (22). Gels were stained with silver (24). Molecular weights were determined by using known proteins as standards.

Two fusions resulted in 133 proliferating hybridomas. One positive anti-97-kDa HmBP MAb-secreting hybridoma was identified by ELISA.

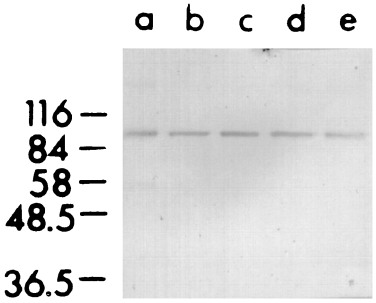

FIG. 1. Immunoblot analysis of the 97-kDa HmBP among *N. gonorrhoeae* strains. Affinity-purified HmBPs were fractionated by SDS-PAGE, and the immunoblot was reacted with the murine anti-97-kDa HmBP MAb. Gonococcal strains represented are PID543 (lane a), PID5 (lane b), W159 (lane c), C439 (lane d), and U750 (lane e). Positions of protein standards in kilodaltons are indicated on the left.

By an immunodot assay, the MAb present in the murine ascites fluid was isotyped as an IgM MAb (data not shown). The IgM affinity purification elution profile of the ascites fluid exhibited a single protein peak, consisting of a homogeneous antibody preparation as assessed by SDS-PAGE (data not shown). Similar results were obtained following affinity purification of the control MAb preparation, an IgM MAb (42-9-6-5) raised against the meningococcal lactoferrin-binding protein (data not shown). Both affinity-purified MAbs were reactive in immunodot analyses against total membranes derived from cells grown under iron-limited conditions and whole cells of *N. gonorrhoeae* PID543 grown under iron-restrictive conditions (data not shown).

When a Western blot of total membranes prepared from cells grown under iron-limited conditions (data not shown) and affinity-isolated HmBPs (Fig. 1, lane a) of *N. gonorrhoeae* PID543 was probed with the anti-97-kDa HmBP MAb, an immunoreactive band corresponding to the 97-kDa HmBP was detected in both preparations, a result indicating that the functional specificity of the anti-97-kDa HmBP MAb was preserved following affinity purification from ascites fluid. The anti-97-kDa HmBP MAb did not react with total membranes derived from gonococcal cultures grown under iron-replete conditions (data not shown).

In an immunoblot analysis of HmBPs affinity purified from a limited survey of clinical gonococcal isolates, the anti-97-kDa HmBP MAb recognized the homologous 97-kDa HmBP in all the isolates (Fig. 1, lanes a to e), signifying that an epitope of the 97-kDa HmBP is conserved among gonococcal strains.

When bovine hemin was supplied as the sole exogenous iron source to iron-restricted gonococcal cultures, the addition of the anti-97-kDa HmBP MAb inhibited growth in a concentration-dependent manner. No growth inhibition was seen in control iron-restricted cultures that did not contain the anti-97-kDa HmBP MAb (Fig. 2A). In addition, gonococcal cultures containing the antilactoferrin control IgM MAb, at a concentration that completely suppressed growth with the anti-97-kDa HmBP MAb, retained the ability to use hemin (Fig. 2A). These results strongly suggest that the anti-97-kDa HmBP MAb interfered with the capacity of the gonococcus to use hemin as an iron source and that this effect was specific to the anti-97-kDa HmBP MAb. The latter result also demonstrates that the growth-inhibitory effects of the anti-97-kDa HmBP MAb are not related to immunoglobulin isotype, in which the binding of the MAb to the 97-kDa HmBP might sterically hinder the interaction of hemin with an adjacent bacterial surface structure involved in hemin uptake.

The anti-97-kDa HmBP MAb, at a concentration that completely suppressed the ability of gonococci to use hemin as an iron source, was also capable of inhibiting growth in which 3 μM human hemoglobin was supplied as the iron source (Fig. 2B). No growth inhibition was seen in the presence of the control MAb (Fig. 2B). The addition of 12 μM human serum albumin to the hemoglobin-containing cultures did not inhibit growth (data not shown). As human serum albumin binds free hemin, the addition of this compound permitted the distinction of growth due to hemoglobin from growth due to hemin that had dissociated from hemoglobin. *N. gonorrhoeae* is incapable of using hemin complexed to albumin as an iron source (10).

The growth inhibition imposed by the anti-97-kDa HmBP MAb was specific for hemin and hemoglobin since the anti-97-kDa HmBP MAb, at a concentration that inhibited the growth-promoting effects of both heme-iron and hemoglobin, did not inhibit the ability of iron-loaded human lactoferrin to donate its iron to support the growth of iron-starved gonococci (data not shown). This result also indicates that the growth-inhibitory effects of the anti-97-kDa HmBP MAb are unlikely to be due to cell agglutination.

To determine the mechanism whereby the anti-97-kDa HmBP MAb interrupted gonococcal hemin use, competitive binding assays were performed. The anti-97-kDa HmBP MAb

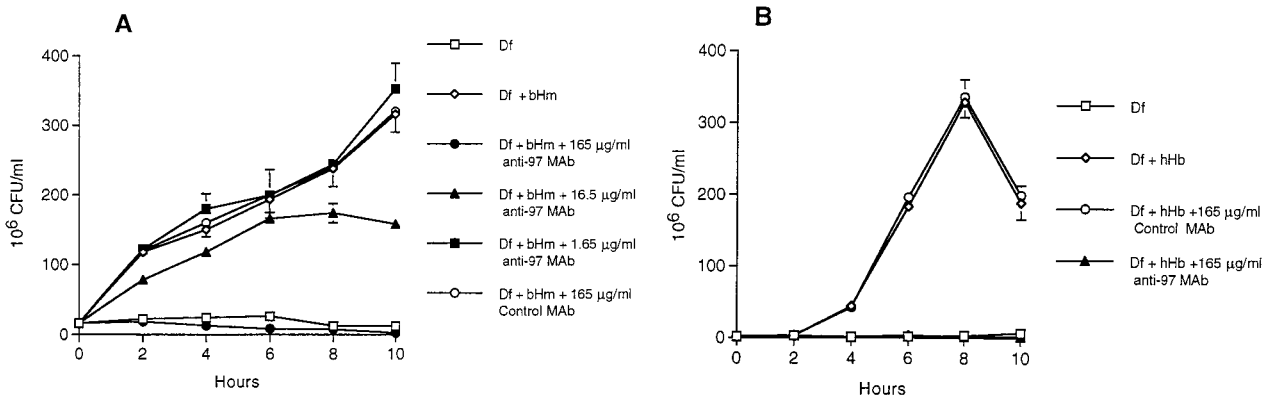

FIG. 2. Growth inhibition of *N. gonorrhoeae* PID543. (A) Either the anti-97-kDa HmBP MAb or the control MAb was added at the indicated concentrations to iron-restricted SJ-GC medium in which 12 μM bovine hemin (bHm) served as the sole source of exogenous iron. (B) The anti-97-kDa HmBP MAb or the control MAb was added to iron-deficient gonococcal cultures in which 3 μM human hemoglobin (hHb) served as the iron source. All gonococcal cultures were rendered iron deficient with the addition of the iron chelator desferrioxamine (Df).

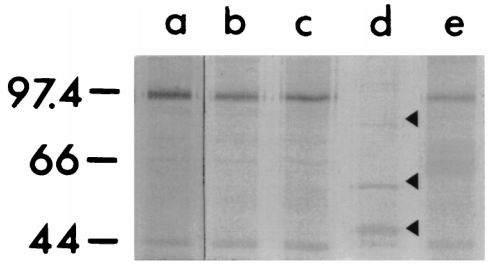

FIG. 3. Binding inhibition of the gonococcal HmBPs by the anti-97-kDa HmBP MAb. The anti-97-kDa HmBP MAb at 0 (lane a), 0.02365 (lane b), 0.2365 (lane c), and 2.365 (lane d) mg/ml and the control MAb at 2.365 mg/ml (lane e) were incubated at 35°C for 1 h with 0.25 mg of total membrane prepared from *N. gonorrhoeae* PID543 grown under iron-deficient conditions. The HmBPs from these preparations were affinity purified and separated by SDS-PAGE. The resultant gel was stained with silver. Sizes of protein standards (in kilodaltons) are shown on the left. Arrowheads indicate additional protein bands of 84, 55, and 47 kDa derived from the anti-97-kDa HmBP IgM MAb.

abrogated binding of the 97-kDa HmBP to the matrix-bound hemin at a MAb concentration of 2.365 mg/ml (Fig. 3, lanes b to d). In contrast, no binding inhibition was detected with the control MAb (Fig. 3, lane e).

The identity of the additional protein bands of 84, 55, and 47 kDa seen in Fig. 3, lane d, was determined by subjecting an identical competitive binding assay gel to Western blotting. Probing of the electroblotted proteins with an HRP-conjugated rabbit anti-mouse IgM antibody revealed immunoreactive bands corresponding to the molecular weights of these proteins that were present only in the affinity-purified samples that contained the anti-97-kDa HmBP MAb (data not shown). Identical bands were seen when an electroblotted sample containing only the anti-97-kDa HmBP MAb was reacted with the HRP-conjugated anti-mouse IgM antibody (data not shown). These results indicate that these proteins are derived from the anti-97-kDa HmBP IgM MAb.

The initial step in hemin acquisition in pathogenic members of the family *Neisseriaceae* has been postulated to involve the specific interaction of hemin with a surface-exposed outer membrane protein(s) (16). The existence of a hemin receptor is supported by biochemical and genetic data. HmBPs are expressed in *N. gonorrhoeae* (14) and in *Neisseria meningitidis* (15). The binding of radiolabelled hemin to the surface of the gonococcus occurs in a specific and saturable manner (9). In the meningococcus, mutants deficient in the hemoglobin receptors HmbR and HpuB retain the ability to use heme as an iron source (27, 28). Moreover, in two other gram-negative bacteria, *Vibrio cholerae* (12) and *Yersinia enterocolitica* (26), the requirement for an outer membrane receptor for hemin acquisition has been established by genetic investigations.

This study provides evidence implicating the involvement of the 97-kDa HmBP in hemin acquisition in *N. gonorrhoeae* and suggests that the 97-kDa HmBP functions as a gonococcal hemin receptor. Such a conclusion is supported by several lines of evidence. An affinity-purified IgM murine MAb specific for the 97-kDa HmBP succeeded in attenuating the growth-promoting effects of hemin. No such growth retardation was witnessed with a similarly affinity-isolated murine IgM MAb which reacted with another gonococcal iron-regulated protein. Competitive binding assays suggested that the inhibitory effect of the anti-97-kDa HmBP MAb on hemin use resides in the ability of the MAb to inhibit binding of 97-kDa HmBP to hemin. In contrast, the control murine MAb did not affect the binding of hemin to the 97-kDa HmBP. However, direct proof of the involvement of the 97-kDa HmBP in hemin transport awaits the construction of the appropriate genetic mutant.

The ability of the anti-97-kDa HmBP MAb to inhibit hemoglobin use suggests that the 97-kDa HmBP may play an essential role in hemoglobin utilization. Consistent with this supposition is the prior demonstration that in competitive binding assays the gonococcal HmBPs recognize the heme moiety of human hemoglobin (14). In *N. meningitidis*, hemoglobin use has been shown to be mediated by two functionally independent hemoglobin receptors, termed HpuB (4a, 18) and HmbR (27). These receptors are distinguished from the meningococcal HmBPs by differences in functional specificity. Similar to the gonococcal HmBPs, the meningococcal HmBPs bind to the heme prosthetic group in hemoglobin (15), whereas the hemoglobin receptors appear to bind to residues in the globin chains of hemoglobin (28). The presence of gonococcal *hmbR* (28) and *hpuB* (4a) homologs suggests that analogous hemoglobin uptake systems are present in the gonococcus. Therefore, hemoglobin uptake in the pathogenic *Neisseriaceae* likely requires the participation of the 97-kDa HmBP and a cognate receptor for hemoglobin. Further support for this suggestion of the involvement of two proteins is provided by Scatchard plot analysis of the binding of biotinylated human hemoglobin to *N. meningitidis* (16a). The binding kinetics are compatible with a two-component model. However, the precise functional significance of the 97-kDa HmBP in the gonococcal hemoglobin uptake pathway remains to be determined.

After binding to its receptor, hemin may be transported intact across the cell envelope into the cytoplasm, as has been shown for *Haemophilus influenzae* type b (8) and *Porphyromonas gingivalis* (11). Alternatively, the iron might be removed from the porphyrin ring prior to entry into the cytoplasm. Results from a recent investigation suggest that this latter mechanism is operative in the gonococcus (9). How this process might be accomplished is uncertain, however, as no periplasmic heme-degrading enzyme has yet been identified in gram-negative bacteria (27). Although this issue was not the objective of the current investigation, an examination of the ability of the anti-97-kDa HmBP MAb to inhibit the binding and uptake of radiolabelled hemin may assist in clarifying the outcome of receptor-bound hemin in the gonococcus. These studies are underway.

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REFERENCES

1. **Anderson, J. E., P. F. Sparling, and C. N. Cornelissen.** 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. J. Bacteriol. **176:**3162–3170.
2. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
3. **Cates, W., J. N. Wasserheit, and P. A. Marchbanks.** 1994. Pelvic inflammatory disease and tubal infertility—the preventable conditions. Hum. Reprod. Ecol. **709:**179–195.
4. **Cates, W., Jr., R. T. Rolfs, Jr., and S. O. Aral.** 1990. Sexually transmitted diseases, pelvic inflammatory disease, and infertility: an epidemiologic update. Epidemiol. Rev. **12:**199–220.

4a. **Chen, C.-J., P. F. Sparling, L. A. Lewis, D. W. Dyer, and C. Elkins.** 1996. Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*. Infect. Immun. **64:**5008–5014.

5. **Cohen, M. S., and P. F. Sparling.** 1992. Mucosal infection with *Neisseria gonorrhoeae*: bacterial adaptation and mucosal defenses. J. Clin. Invest. **89:** 1699–1705.
6. **Cornelissen, C. N., G. D. Biswas, and P. F. Sparling.** 1993. Expression of gonococcal transferrin-binding protein 1 causes *Escherichia coli* to bind human transferrin. J. Bacteriol. **175:**2448–2450.
7. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
8. **Coulton, J. W., and J. C. S. Pang.** 1983. Transport of hemin by *Haemophilus influenzae* type b. Curr. Microbiol. **9:**93–98.
9. **Desai, P. J., R. Nzeribe, and C. A. Genco.** 1995. Binding and accumulation of hemin in *Neisseria gonorrhoeae*. Infect. Immun. **63:**4634–4641.
10. **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.
11. **Genco, C. A., B. M. Odusanya, and G. Brown.** 1994. Binding and accumulation of hemin in *Porphyromonas gingivalis* are induced by hemin. Infect. Immun. **62:**2885–2892.
12. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461–469.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Lee, B. C.** 1992. Isolation of heme-binding proteins of *Neisseria gonorrhoeae*. J. Med. Microbiol. **36:**121–127.
15. **Lee, B. C.** 1994. Isolation and characterization of the haemin-binding proteins from *Neisseria meningitidis*. Microbiology **140:**1473–1480.
16. **Lee, B. C.** 1995. Quelling the red menace: heme capture by bacteria. Mol. Microbiol. **18:**383–390.

16a. **Lee, B. C., and P. Hill.** 1992. Identification of an outer membrane haemoglobin-binding protein in *Neisseria meningitidis*. J. Gen. Microbiol. **138:**2647–2656.

17. **Lee, B. C., and A. B. Schryvers.** 1988. Specificity of the lactoferrin and transferrin receptors in *Neisseria gonorrhoeae*. Mol. Microbiol. **2:**827–829.
18. **Lewis, L. A., and D. W. Dyer.** 1995. Identification of an iron-regulated outer membrane protein of *Neisseria meningitidis* involved in the utilization of hemoglobin complexed to haptoglobin. J. Bacteriol. **177:**1299–1306.
19. **Longenecker, B. M., T. R. Mosmann, and C. Shiozawa.** 1979. A strong, preferential response of mice to polymorphic antigenic determinants of the chicken MHC, and analyzed with mouse hybridoma (monoclonal) antibodies. Immunogenetics **9:**137–147.
20. **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.
21. **Mackie, E. B., L. A. White, K. N. Brown, and L. E. Bryan.** 1981. Enzyme-linked immunosorbent assay compared with immunoprecipitation for serotyping *Neisseria meningitidis* and its use in demonstrating serotype polymorphism. J. Clin. Microbiol. **13:**449–455.
22. **Martel, N., and B. C. Lee.** 1993. The acquisition of heme-iron in *Neisseria meningitidis* does not involve the meningococcal transferrin-binding proteins. Infect. Immun. **62:**700–703.
23. **McKenna, W. R., P. A. Mickelsen, P. F. Sparling, and D. W. Dyer.** 1988. Iron uptake from lactoferrin and transferrin by *Neisseria gonorrhoeae*. Infect. Immun. **56:**785–791.
24. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105:**361–363.
25. **Shockley, R. K., E. E. Coffee, and K. H. Johnston.** 1980. SJ-GC, a modified complete medium for the growth of *Neisseria gonorrhoeae*. J. Clin. Microbiol. **12:**35–38.
26. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J. **11:**4359–4367.
27. **Stojiljkovic, I., V. Hwa, L. de Saint Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. Mol. Microbiol. **15:**531–541.
28. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anjic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670–4678.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
30. **Tsutsui, K., and G. C. Mueller.** 1982. Affinity chromatography of heme-binding proteins: an improved method for the synthesis of hemin-agarose. Anal. Biochem. **121:**244–250.

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