Conserved Lipoproteins of Pathogenic *Neisseria* Species Bearing the H.8 Epitope: Lipid-Modified Azurin and H.8 Outer Membrane Protein

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Many of the surface components of the pathogenic *Neisseria* species demonstrate marked antigenic variability, both between different strains and among variants of a single strain. Numerous examples of such variable surface components are described in the other reviews in this issue. Amid this surface variability, there are relatively few components or antigens that are conserved among all gonococci or meningococci, or both. The conserved antigens that have been identified have been the subject of considerable study in an effort to determine whether they play a direct role in neisserial pathogenesis and whether they might be targets of a protective immune response. The existence of a protein epitope that is conserved among pathogenic *Neisseria* species was revealed by the binding of a monoclonal antibody (MAb) designated H.8 (8), as well as other MAbs with similar specificities (14, 31). These MAbs bind to all gonococci and meningococci that have been tested and to *N. lactamica* and *N. cinerea* strains, but not to strains of other commensal *Neisseria* species (1, 8, 14, 31). There has been much recent progress in identifying and characterizing the proteins that are recognized by H.8-specific MAbs, particularly through the use of recombinant deoxyribonucleic acid (DNA) approaches. The existence of multiple proteins that bind H.8-specific MAbs was revealed by screening libraries of gonococcal and meningococcal genes. Gotschlich et al. (11) cloned two distinct gonococcal genes that hybridize to each other in Southern blotting experiments and encode H.8 MAb-binding proteins with similar apparent molecular weights. Further analysis of these and other cloned genes has added to our understanding of the characteristics of two different proteins that are recognized by H.8-specific MAbs: the lipid-modified azurin and the H.8 outer membrane protein (designated Laz and Lip, respectively, in the nomenclature recommendations included in this issue).

LIPID-MODIFIED AZURIN (Laz)

The Laz protein is a two-domain lipoprotein that is highly conserved in gonococci and meningococci. The gene encoding Laz has been cloned and sequenced from both a gonococcal (11, 12) and a meningococcal (17) strain; the two genes are virtually identical. The DNA sequence of the *laz* gene predicts that the protein has the following features. (i) The first is a most probable signal peptide-processing site matching the consensus site for bacterial lipoproteins. In other procaryotic lipoproteins, this site is recognized and processed by signal peptidase II, resulting in an N-terminal cysteine residue modified with glycerol and fatty acid (21, 30). The activity of signal peptidase II is specifically inhibited by the cyclic peptide antibiotic globomycin (15). Using the cloned meningococcal *laz* gene in *Escherichia coli*, we showed that the Laz protein could be modified with radio-labeled palmitic acid and that its processing was sensitive to

inhibition by globomycin, thus confirming the similarity of the recombinant-derived neisserial protein to other procaryotic lipoproteins (J. P. Woods, J. F. Dempsey, T. H. Kawula, D. S. Barritt, and J. G. Cannon, Mol. Microbiol., in press).

(ii) The predicted mature Laz protein has a molecular mass of 17 kilodaltons. The N-terminal 39 amino acids of the mature protein form a domain composed primarily of imperfect repeats of the sequence alanine-alanine-glutamate-alanine-proline (AAEAP). The epitope for H.8 MAb binding was localized to no more than 20 amino acids within this region by analysis of deletion subclones of the meningococcal *laz* gene and by analysis of MAb binding to synthetic peptides (17). Thus, the epitope for binding of the H.8 MAb is linear and is not dependent on the covalent lipid modification of the protein. There is little or no visible Laz signal when Western immunoblots of gonococci or meningococci are probed with the H.8 MAb, suggesting that the binding of the MAb to Laz is below the threshold of detection by this technique (11; Woods et al., in press). The presence of the H.8 epitope on Laz was revealed only when the genes were cloned on plasmid (17) or bacteriophage (11) vectors, thus amplifying signal intensity.

(iii) The remainder of the Laz protein (127 amino acids) has striking similarity to the sequence of azurins, which are small, blue, copper-containing proteins that are believed to function in electron transport during respiration (10, 16, 25, 26). Azurins from *Pseudomonas*, *Alcaligenes*, and *Bordetella* species show considerable amino acid sequence homology, including 48 of 129 amino acids that are completely conserved among nine sequenced azurins (26). When the 127 C-terminal acids of Laz are compared with a composite azurin sequence, 77% of the amino acids are homologous. Of the 48 conserved azurin amino acids, 42 (88%) are identical in the predicted Laz protein, including the 4 azurin amino acids forming the copper-binding site (22). The azurins from other bacterial genera are not lipoproteins and do not have the 39-amino-acid N-terminal domain that is present in the neisserial Laz protein (9, 25, 26).

In addition to DNA sequence similarity, there is other evidence confirming the similarity of the neisserial Laz protein to azurins. Gonococcal Laz has been purified from an *E. coli* lysogen containing the cloned *laz* gene, and the spectral characteristics of the resulting blue protein are the same as those reported for conventional azurins (E. Gotschlich, personal communication). Also, the gonococcal and meningococcal Laz proteins cross-react with antiserum to purified *Pseudomonas* azurin. Commensal *Neisseria* species also produce an azurinlike protein recognized by the anti-azurin serum. The characteristics of the azurins from commensal species have not been completely determined, but the proteins may differ in some features from the Laz of the pathogens (Woods et al., in press).

What is the function of the Laz protein? In other bacterial genera, azurins function in electron transfer, most probably between cytochrome c_{551} and cytochrome oxidase (10, 25). Gotschlich and Seiff (12) suggested that the azurinlike domain of Laz might function in electron transport during anaerobic growth of gonococci or meningococci, which can occur if nitrite is provided as a terminal electron acceptor (18). If the Laz protein does play such a role, a mutant lacking Laz might be expected to be unable to grow under anaerobic conditions. Gotschlich has constructed such a mutant of gonococcal strain F62 by insertionally inactivating the cloned *laz* gene and introducing the mutated gene by transformation into the gonococcus. The Laz^- mutants grow under both aerobic and anaerobic conditions in the presence of nitrite (Gotschlich, personal communication). It is still possible that Laz functions in electron transport via a pathway that has not yet been identified in gonococci or meningococci. Since both pathogenic and commensal *Neisseria* species produce Laz or a protein similar to it, it seems unlikely that Laz will have a specific role in enhancing the virulence of gonococci and meningococci.

H.8 OUTER MEMBRANE PROTEIN (Lip)

When gonococcal or meningococcal outer membrane preparations are subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with H.8-specific MABs in a Western blot, a MAB-binding band with unusual electrophoretic characteristics is observed. On one-dimensional gels, the antigen forms a cone-shaped band with an apparent molecular mass ranging from 18 to 30 kilodaltons (8, 14, 17). On two-dimensional gels, the antigen migrates away from diagonal (14). The protein responsible for this MAB-binding signal, designated the H.8 outer membrane protein, H.8 antigen, or Lip (for lipoprotein), does not stain with Coomassie blue and stains with silver only under some conditions (2, 8, 14). The apparent molecular mass of Lip varies in different strains, but appears to be constant within a single gonococcal or meningococcal strain (8, 14).

Two different methods for the purification of Lip have been published. Strittmatter and Hitchcock (28) used phenol-chloroform-petroleum ether extraction of gonococci, followed by several chromatographic steps, to obtain purified Lip. The protein copurifies with two lipid components, is alanine and proline rich, and lacks aromatic amino acids and methionine. Bhattacharjee et al. (2) purified meningococcal Lip by Empigen BB extraction and high-performance liquid chromatography. The purified protein is rich in glutamate, alanine, and proline and contains a lipid component that elutes between C_9 and C_{11} straight-chain fatty acid standards in the gas chromatograph. The unusual amino acid composition of the protein is consistent with its lack of absorbance at 280 nm (2, 28). The pattern of biosynthetic labeling of Lip with radiolabeled amino acids and fatty acids is consistent with the composition determined for purified Lip protein (W. Baehr, E. C. Gotschlich, and P. J. Hitchcock, Mol. Microbiol., in press).

Recent studies on the cloning and sequencing of gonococcal *lip* genes have clarified the structure of this unusual protein. Gotschlich et al. (11) produced two different λ gt11 clones containing the *lip* gene of gonococcal strain R10, and those clones have been sequenced (Baehr et al., in press). The *lip* gene from strain FA1090 was cloned in a lambda bacteriophage vector (5) and sequenced by Woods et al. (J. P. Woods, S. M. Spinola, S. M. Strobel, and J. G. Cannon, Mol. Microbiol., in press). The DNA sequence of the

lip gene in the two strains is quite similar. In each case, the DNA sequence predicts that Lip is a lipoprotein, with a lipoprotein signal peptide-processing site like that of the Laz protein. The N-terminal amino acid of mature Lip protein in both strains is a cysteine residue. In FA1090, the predicted protein is 71 amino acids in length, composed entirely of 13 repeats of the AAEAP consensus sequence that was also found in the epitope-encoding region of Laz (12, 17). Perfect five-residue periodicity is maintained throughout the protein. Seven of the repeats match the consensus sequence exactly, five have single amino acid substitutions, and one has two amino acid substitutions. Only six of the amino acids in the mature protein are not included in the repetitive sequence. In strain R10, the predicted protein is 76 amino acids long, consisting of 14 AAEAP repeats. Ten of the repeats are perfect. It is possible that differences in the number of AAEAP repeats contribute to the differences in apparent molecular mass of Lip in different strains. Despite the high proline content of the protein, the predicted secondary structure is α -helical. The predicted molecular mass of the polypeptide portion of FA1090 Lip is 6.3 kilodaltons but the protein migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent molecular mass of 20 kilodaltons. The aberrant migration of the protein may be due to its lipid modification or to its unusual amino acid composition and repeating structure. The sequence of a meningococcal *lip* gene has not yet been determined.

Although Lip is noteworthy in the perfect conservation of most of the AAEAP repeats, there are other microbial proteins with similar repetitive motifs. Streptococcal M protein contains imperfect heptapeptide repeats making up part of the protein. The repetitive nature of this region is important in formation of an α -helical coiled-coil structure and in the antiphagocytic function of the protein (19, 23). Another protein with a repetitive structure is the major lipoprotein (Lpp) of *E. coli*, which has several intriguing similarities to gonococcal Lip. In both proteins, the polypeptide is relatively small (58 amino acids for Lpp) and has an N-terminal cysteine and a C-terminal lysine residue (21, 30). The linkage of Lpp to peptidoglycan is through the ϵ -amino group of the C-terminal lysine (7). In Lpp, a seven-amino-acid periodicity is important in the formation of the characteristic coiled-coil structure of the protein (6, 20). The seven-amino-acid repeats in the *E. coli* Lpp are not as highly conserved as the AAEAP repeats in Lip. It is not known whether Lip serves a structural role in the outer membrane similar to that of Lpp or whether it is peptidoglycan associated under some or all growth conditions.

ROLE OF H.8 (Lip) IN NEISSERIAL PATHOGENESIS AND IMMUNITY

The association of the H.8 epitope with the pathogenic *Neisseria* species has led to speculation that the antigens bearing it might be involved in neisserial pathogenesis or that they might be effective as components of a vaccine for prevention of gonococcal or meningococcal disease. The majority of H.8 MAB binding is to the Lip protein, which consists essentially of a repeating H.8 epitope. There are conflicting data about whether the epitope recognized by the MAB is exposed on the surface of intact, viable organisms. For gonococci, experiments involving adsorption of MAB from solution (8), fluorescent-antibody labeling (14), slide agglutination (14), and immunoelectron microscopy (14) have been interpreted as evidence for surface exposure. However, other studies involving immunoelectron micros-

copy (24) showed that the majority of organisms do not mark with gold-conjugated MAb, although outer membrane blebs label heavily. In any of these studies, the apparent surface exposure of the target epitope could be influenced by the conditions used for preparation and fixation of the cells, and it is difficult to draw firm conclusions about this issue. Lip is immunogenic in patients with localized or disseminated gonococcal infections (3, 4, 13) and in patients with meningococcal disease (3). It is not known whether an antibody response directed at Lip would protect against infection. In the mouse model of meningococcal infection, MAb specific for Lip was not protective (29). However, the MAb used in those experiments was not bactericidal, leaving the possibility open that a Lip-specific MAb would be protective. Also, Schweinle et al. have obtained data indicating that MAb 10, which is specific for the H.8 epitope, is bactericidal and opsonic for some gonococcal strains (J. E. Schweinle, P. J. Hitchcock, A. J. Tenner, C. H. Hammer, M. M. Frank, and K. A. Joiner, J. Clin. Invest., in press).

The recent construction of a gonococcal mutant lacking the Lip protein should allow better determination of the role of this protein in neisserial physiology and virulence (Woods et al., in press). In this mutant, the gene encoding Lip was insertionally inactivated by using the shuttle mutagenesis system developed for gonococci by Seifert et al. (27). The Lip-less mutant shows no alteration in its growth rate in rich medium and is unaltered in its serum resistance.

It may be necessary to reevaluate the available data about the association of Lip with pathogenic *Neisseria* species in light of recent information gained about the structure of the protein. When commensal *Neisseria* species are probed with H.8-specific MAbs or with DNA probes derived from the *lip* gene, they are negative for MAb binding or DNA hybridization (1, 5, 8). However, since Lip consists only of repeats of a single unit, encoded by a similarly repeating DNA sequence, it is possible that commensal *Neisseria* species have a different protein with a slightly different repeat unit. Such a protein would not be detected by the MAb and DNA probes derived from *lip* of the pathogens. The association of the H.8 epitope with pathogenic *Neisseria* species may be a reflection of clonal relationships among *Neisseria* species, rather than a consequence of a direct role of the protein in pathogenesis. The question of the role of Lip in gonococcal or meningococcal pathogenesis is still open, and further experiments are needed to clarify the function of this unusual lipoprotein in pathogenic *Neisseria* species.

LITERATURE CITED

- Aho, E. L., G. L. Murphy, and J. G. Cannon. 1987. Distribution of specific DNA sequences among pathogenic and commensal *Neisseria* species. *Infect. Immun.* **55**:1009-1013.
- Bhattacharjee, A. K., E. E. Moran, J. S. Ray, and W. D. Zollinger. 1988. Purification and characterization of H.8 antigen from group B *Neisseria meningitidis*. *Infect. Immun.* **56**:773-778.
- Black, J. R., W. J. Black, and J. G. Cannon. 1985. Neisserial antigen H.8 is immunogenic in patients with disseminated gonococcal and meningococcal infections. *J. Infect. Dis.* **151**:650-657.
- Black, J. R., M. K. Thompson, J. G. Cannon, C. Lammel, and G. F. Brooks. 1988. Serum immune response to common pathogenic *Neisseria* antigen H.8 in patients with uncomplicated gonococcal infection and pelvic inflammatory disease, p. 493-498. *In* J. T. Poolman, H. C. Zanen, T. F. Meyer, J. E. Heckels, P. R. H. Makela, H. Smith, and E. C. Beuvery (ed.), *Gonococci and meningococci*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Black, W. J., and J. G. Cannon. 1985. Cloning of the gene for the common pathogenic *Neisseria* H.8 antigen from *Neisseria gonorrhoeae*. *Infect. Immun.* **47**:322-325.
- Braun, V., and V. Bosch. 1972. Repetitive sequences in the murein-lipoprotein of the cell wall of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **69**:970-974.
- Braun, V., and U. Sieglin. 1970. The covalent murein-lipoprotein structure of the *Escherichia coli* cell wall. *Eur. J. Biochem.* **13**:336-346.
- Cannon, J. G., W. Black, I. Nachamkin, and P. Stewart. 1985. Monoclonal antibody that recognizes an outer membrane antigen common to pathogenic *Neisseria* species but not to most nonpathogenic *Neisseria* species. *Infect. Immun.* **43**:994-999.
- Canter, G. W. 1987. The azurin gene from *Pseudomonas aeruginosa* codes for a pre-protein with a signal peptide. *FEBS Lett.* **212**:168-172.
- Farver, O., and I. Pecht. 1984. The reactivity of copper sites in the "blue" copper proteins, p. 183-214. *In* R. Lontie (ed.), *Copper proteins and copper enzymes*. CRC Press, Inc., Boca Raton, Fla.
- Gotschlich, E. C., M. S. Blake, J. M. Koomey, M. Seiff, and A. Derman. 1986. Cloning of the structural genes of three H.8 antigens and of protein III of *Neisseria gonorrhoeae*. *J. Exp. Med.* **164**:868-881.
- Gotschlich, E. C., and M. E. Seiff. 1987. Identification and gene structure of an azurin-like protein with a lipoprotein signal peptide in *Neisseria gonorrhoeae*. *FEMS Microbiol. Lett.* **43**:253-255.
- Hicks, C. B., J. W. Boslego, and B. Brandt. 1987. Evidence of serum antibodies to *Neisseria gonorrhoeae* before gonococcal infection. *J. Infect. Dis.* **155**:1276-1281.
- Hitchcock, P., S. Hayes, L. Mayer, W. Schafer, and S. Tessier. 1985. Analysis of the gonococcal H.8 antigen: surface location, inter- and intra-strain electrophoretic heterogeneity and unusual two-dimensional electrophoretic characteristics. *J. Exp. Med.* **162**:2017-2034.
- Hussain, M., S. Ichihara, and S. Mizushima. 1980. Accumulation of glyceride-containing precursor of the outer membrane lipoprotein in the cytoplasmic membrane of *Escherichia coli* treated with globomycin. *J. Biol. Chem.* **255**:3707-3712.
- Kakutani, T., H. Watanabe, K. Arima, and T. Beppu. 1981. A blue protein as an inactivating factor for nitrite reductase from *Alcaligenes faecalis* strain S-6. *J. Biochem.* **89**:463-472.
- Kawula, T. H., S. M. Spinola, D. G. Klapper, and J. G. Cannon. 1987. Localization of a conserved epitope and an azurin-like domain in the H.8 protein of pathogenic *Neisseria*. *Mol. Microbiol.* **1**:179-185.
- Knapp, J. S., and V. L. Clark. 1984. Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. *Infect. Immun.* **46**:176-181.
- Manjula, B. N., and V. A. Fischetti. 1980. Tropomyosin-like seven residue periodicity in three immunologically distinct streptococcal M proteins and its implications for the antiphagocytic property of the molecule. *J. Exp. Med.* **151**:695-708.
- McLachlan, A. D. 1978. The double helix coiled coil structure of murein lipoprotein from *Escherichia coli*. *J. Mol. Biol.* **122**:493-506.
- Mizushima, S. 1984. Post-translational modification and processing of outer membrane prolipoproteins in *Escherichia coli*. *Mol. Cell. Biochem.* **60**:5-15.
- Norris, G. E., B. F. Anderson, and E. N. Baker. 1983. Structure of azurin from *Alcaligenes denitrificans* at 2.5 angstrom resolution. *J. Mol. Biol.* **165**:501-521.
- Phillips, G. N., Jr., P. F. Flicker, C. Cohen, B. N. Manjula, and V. A. Fischetti. 1981. Streptococcal M protein: α -helical coiled-coil structure and arrangement on the cell surface. *Proc. Natl. Acad. Sci. USA* **78**:4689-4693.
- Robinson, E. N., Jr., Z. A. McGee, T. M. Buchanan, M. S. Blake, and P. J. Hitchcock. 1987. Probing the surface of *Neisseria gonorrhoeae*: simultaneous localization of protein I and H.8 antigens. *Infect. Immun.* **55**:1190-1197.
- Ryden, L. 1984. Structure and evolution of the small blue proteins, p. 157-182. *In* R. Lontie (ed.), *Copper proteins and*

- copper enzymes. CRC Press, Inc., Boca Raton, Fla.
26. Ryden, L., and J.-O. Lundgren. 1976. Homology relationships among the small blue proteins. *Nature (London)* **261**:344-346.
 27. Seifert, H. S., M. So, and F. Heffron. 1986. Shuttle mutagenesis: a method of introducing transposons into transformable organisms, p. 123-134. *In* J. K. Setlow and A. Hollaender (ed.), *Genetic engineering, principles and methods*. Plenum Publishing Corp., New York.
 28. Strittmatter, W., and P. J. Hitchcock. 1986. Isolation and preliminary biochemical characterization of the gonococcal H.8 antigen. *J. Exp. Med.* **164**:2038-2048.
 29. Woods, J. P., J. R. Black, D. S. Barritt, T. D. Connell, and J. G. Cannon. 1987. Resistance to meningococcemia apparently conferred by anti-H.8 monoclonal antibody is due to contaminating endotoxin and not to specific immunoprotection. *Infect. Immun.* **55**:1927-1928.
 30. Wu, H. 1987. Posttranslational modification and processing of membrane proteins in bacteria, p. 37-71. *In* M. Inouye (ed.), *Bacterial outer membranes as model systems*. John Wiley & Sons, Inc., New York.
 31. Zollinger, W., J. Ray, E. Moran, and R. Seid. 1985. Identification by monoclonal antibody of an antigen common to the pathogenic *Neisseria* species, p. 579-584. *In* G. Schoolnik, G. F. Brooks, S. Falkow, C. E. Frasch, J. S. Knapp, J. A. McCutchan, and S. A. Morse (ed.), *The pathogenic neisseriae*. American Society for Microbiology, Washington, D.C.