ISOLATION AND PRELIMINARY BIOCHEMICAL
CHARACTERIZATION OF THE GONOCOCCAL
H.8 ANTIGEN

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During the last two decades, considerable efforts have been made to identify gonococcal surface constituents and to study their relevance in the host bacterium interaction. With the introduction of monoclonal antibodies, previously undetected neisserial antigens with M_r 22,000–27,000 have been discovered and described (1–4). These antigens have several common properties: they do not stain with Coomassie, they are all identified by their reaction with specific mAb, and they show a strain-specific variability in electrophoretic mobility (1–4). In this laboratory, comparative studies using the H.8 monoclonal (1) and mAb 10 led to the conclusion that both mAbs recognize the same gonococcal antigen (2).

The H.8 antigen is particularly interesting because it is found almost exclusively on the pathogenic Neisseria (gonococci and meningococci) (1) and because it is surface exposed (2). Studies (5) have demonstrated that the antigen is immunogenic in patients with disseminated gonococcal and meningococcal disease. The M_r of the gonococcal H.8 antigen appears to be invariable within a given strain (2) but different among most strains, however such changes do not affect the binding of mAb 10 to the antigens (1, 2). Recently (6) the genes for both gonococcal and meningococcal H.8 antigens have been cloned.[1]

Further characterization (1, 2) has demonstrated that immunoreactivity of the antigen with polyclonal and monoclonal antibodies is lost upon protease treatment. The H.8 antigen has been refractory to radiolabeling with a number of different isotopes; however, limited success has been achieved using ^{14}C-labeled amino acids (2). The antigen has unusual electrophoretic mobility after separation in two-dimensional SDS-PAGE (2). These electrophoretic characteristics are thought to be indicative of tightly associated lipid or carbohydrate components.

Because H.8 is a complex molecule, we chose to isolate and characterize it using a biochemical approach. The chemical analysis of the purified H.8 antigen will provide information about its biochemical nature and structure and may give us insight into its biological function and its role in the host–bacterium interaction. In this report, we present a purification scheme for the isolation of the gonococcal H.8 antigen and data of preliminary analyses of the purified antigen.

Materials and Methods

Strain and Growth Conditions. *Neisseria gonorrhoeae* strain JS3 (7) was grown on gonococcal clear typing medium (8) in 5% humidified CO_2 atmosphere at 37°C. Several piliation and opacity phenotypes were used for H.8 isolation; the method described was universally applicable to these JS3 colonial variants. 20-h cultures were harvested by gently scraping the organism from the agar surface using a glass rod and distilled water.

Phenol/Chloroform/Petroleum Ether (PCP)[2] Extraction of Gonococcus. Harvested bacteria were dialyzed extensively against distilled water and then dried with successive washes of 200 ml methanol/chloroform/acetone. Dried cells were then extracted three times with a total volume of 45 ml PCP mixture (2:5:8, vol/vol/vol) according to Galanos et al. (9). After petroleum ether and chloroform were completely removed by rotary evaporation, LPS was precipitated from the phenol by adding water. The phenolic fraction containing gonococcal proteins and the H.8 antigen was used for further purification.

Column Chromatography on Sephadex LH20. Phenolic fractions of the PCP extraction were applied to a Sephadex LH20 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (25 × 21 cm) equilibrated with 33% glycerol 6 M urea, and eluted with the same buffer at a flow rate of 0.3 ml/min.

Separation of Chromatofocusing. The LH20 fractions were applied to a chromatofocusing MonoP HR 5/20 fast-protein liquid chromatography (FPLC) column (Pharmacia Fine Chemicals) used in combination with a Waters Model 840 (Waters Associates, Inc., Milford, MA) high-performance liquid chromatography (HPLC). The column was equilibrated with 0.025 M Tris buffer, pH 9.0, containing 1% octyl glucose (10). Eluent for the chromatofocusing chromatography was Servalyt 9-3 HCl (Serva, Feinbiochemica, Heidelberg, Federal Republic of Germany), diluted 1:45, pH 3.0, at a flow rate of 0.5 ml/min.

QAE Chromatography. The chromatofocusing fractions were dialyzed against 0.025 M Tris buffer, pH 9.0 (buffer A), and applied to a MonoQ FPLC column (Pharmacia Fine Chemicals) equilibrated with the same buffer. Chromatography was done by a linear gradient of buffer A and buffer B (0.025 M Tris, pH 9.0, 1 M NaCl) from 5–100% at a flow rate of 1.5 ml/min.

C-8 Reversed-phase Chromatography. In the final step of purification, the fractions obtained by anion-exchange chromatography were separated by a gradient elution from a reversed-phase HPLC C-8 column (Protesil 300 C-8 column; Whatman Inc., Clifton, NJ) at a flow rate of 1 ml/min. To set up the gradient, buffer A (H_2O, 0.1% trifluoroacetic acid [TFA]) was continuously mixed with buffer B (66% acetonitrile, 33% isopropanol, 0.1% TFA) resulting in the following gradient: 0–50% B in 5 min; 50–100% B in 15 min; hold 100% B for 20 min.

Detection of H.8 Antigen. H.8-specific mAb 10 was used to detect the antigen (2) in either immunoelectroblot (11–14) or dot-blot assays (Bio-Rad Laboratories, Richmond, CA). Samples were subjected to SDS-PAGE, electroblotted onto Millipore HAHY 000 10 paper (Millipore Corp., Bedford, MA), incubated with antibodies, and reacted with ^{125}I-labeled protein A (New England Nuclear, Boston, MA). Gels were also stained with either Coomassie Brilliant Blue R250 or silver strain (15). For the dot-blot assay, aliquots of column fractions were loaded into the Millipore paper and probed with mAb 10 followed by ^{125}I-labeled protein A (12), according to directions provided by the manufacturer (Bio-Rad Laboratories).

Amino Acid Analysis. The amino acid composition of the peptide was determined after total hydrolysis (6 N HCl, 105°C, 20 h) using a Pico-Tag (Waters Associates Inc.) system according to procedures supplied by the manufacturer.

Fatty Acid Analysis. Fatty acids were determined as methyl esters by gas/liquid chromatography on a Perkin-Elmer 900 chromatograph (Perkin-Elmer, Norwalk, CT) operated at 190°C, with a capillary 25QC2/BP10.25 column (Scientific Glass Engineering, Austin, TX) comparing their R_f values with those of authentic standards (Supelco, Inc., Bellefonte, PA). Fatty acids were liberated and derivatized by transesterification in 2 N HCl in methanol at 85°C for 16 h (16).

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[2] *Abbreviations used in this paper:* FPLC, fast-protein liquid chromatography; PCP, phenol/chloroform/petroleum ether; TFA, trifluoroacetic acid.
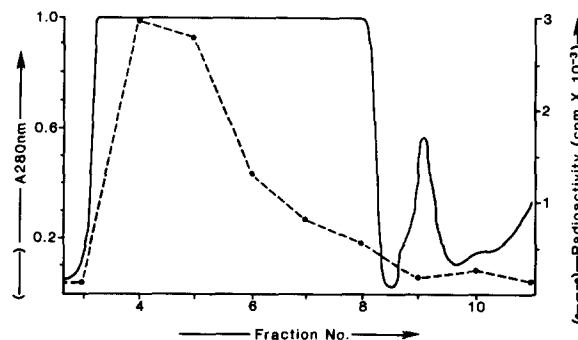
FIGURE 1.   Gel filtration on LH20 Sephadex of phenol-extracted proteins. The material was eluted with glycerol/urea buffer. The absorbance at 280 nm (*solid line*) was monitored, and the reactivity of each fraction with mAb 10 (*dashed line*) was measured by a dot-blot assay.

Criteria for Purity of H.8 Antigen Preparations.   Coomassie- and silver-stained SDS gels were used to detect contaminating gonococcal proteins in the purified H.8 antigen samples. In addition, SDS-PAGE-separated samples were analyzed by immunoelectroblotting using hyperimmune rabbit antiserum to homologous whole gonococci (gift of O. Barrera and J. Swanson, NIH Rocky Mountain Laboratories).

Results

The strategy for extraction of the H.8 antigen was influenced by the observation that a lipid or carbohydrate component might be associated with the antigen. Outer membrane proteins and LPS are closely associated and sometimes can be readily coextracted using the PCP method (14, 17).

PCP Extraction of Gonococci.   The bacteria were extracted as described above. Analysis of the LPS-free phenolic residue by Coomassie Brilliant Blue–stained SDS gels revealed that two proteins were coextracted: an M_r 34,000 (putative protein I) and an M_r 20,000 protein. Examination of extracted whole cells by immunoelectroblotting using mAb 10 showed that, in addition to these proteins, the H.8 antigen was very efficiently extracted by this method. Phenolic residues of the PCP extraction were used for further purification of H.8 antigen (data not shown).

LH20 Column Chromatography.   For the next purification step, LH20 chromatography was chosen to remove the phenol from the crude extract. Phenolic residues of the PCP extraction (6 ml) were applied to the LH20 column, equilibrated with buffer, and eluted. The effluent was monitored for protein at 280 nm and collected as 6-ml fractions (Fig. 1). Aliquots of each fraction were assayed for H.8 content by immunoelectroblotting or by dot-blot assay. The majority of the immunoreactive H.8 antigen eluted in the void volume of the column (fractions 4–6). Together with the other coeluting gonococcal proteins, H.8-containing fractions were collected and equilibrated against 0.025 M Tris buffer, pH 9.0, containing 1% octyl glucoside.

Separation by Chromatofocusing.   Further separation of LH20 fractions was accomplished by using a chromatofocusing FPLC column. The chromatogram is shown in Fig. 2. Two major peaks eluted at pH ~6.0–5.5 and ~5.0–3.0. Both peaks contained proteins that absorbed at 280 nm. The first peak contained an
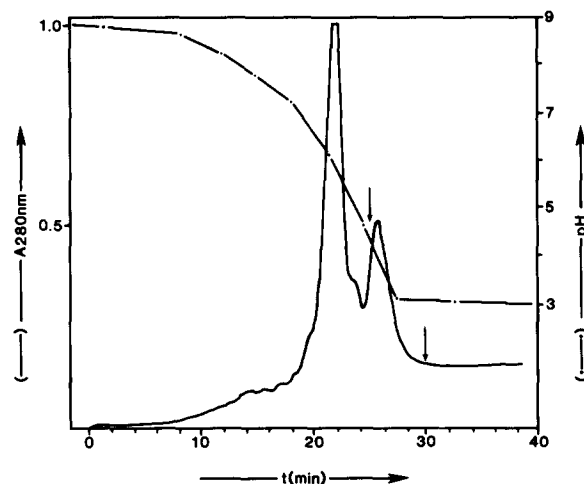
FIGURE 2. Chromatofocusing of LH20 fractions. Pooled H.8$^+$ fractions previously equilibrated with pH 9.0 Tris buffer were eluted in a pH gradient of pH 9.0–3.0 (—●—). Elution was monitored by following 280 nm absorption. H.8-containing fractions (between the two arrows) were identified by western blot analysis.
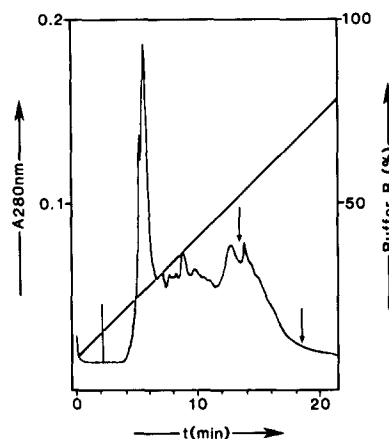

FIGURE 3. QAE column chromatography of H.8 fractions from chromatofocusing. Material was eluted with a linear gradient (solid line) formed by mixing of buffer A (0.025 M Tris HCl, pH 9.0) with buffer B (buffer A, 1 M NaCl). H.8-containing fractions (between the arrows) were identified by western blot analysis.

M_r 34,000 protein. The H.8 antigen eluted in the second peak and was detected in fractions marked by arrows (Fig. 2); analysis of these fractions by SDS-PAGE revealed the presence of Coomassie-staining proteins (data not shown).

QAE Chromatography. Ion-exchange chromatography was used in the subsequent purification of H.8 containing fractions derived from the second peak of the chromatofocusing chromatography. The elution profile of this separation is shown in Fig. 3. A major peak of material absorbing at 280 nm, containing mainly protein I (data not shown), eluted followed by a broad peak. The H.8 antigen eluted at the last part of this peak, at 0.5–0.6 M NaCl, as indicated by the arrows. The material absorbing at 280 nm, which coeluted with the H.8
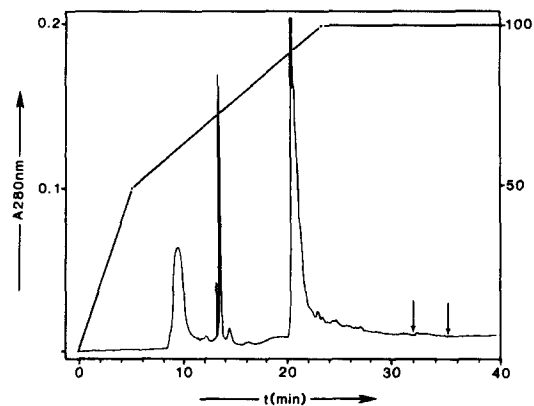
FIGURE 4. Separation of QAE-fractions by reversed-phase HPLC. Linear gradients (composition described above (—●—) were generated by mixing solvent A (H_2O, 0.1% TFA) with solvent B (66% CH_3CN, 33% isopropanol, 0.1% TFA). Elution of protein was monitored by absorption at 280 nm. H.8$^+$ fractions were detected between the two arrows.

antigen, migrated primarily as an M_r 34,000 protein (protein I) in Coomassie-stained polyacrylamide gels (data not shown).

C-8 Reversed-phase Chromatography.   A C-8 column was chosen because preliminary results from experiments using a C-18 column indicated the retention time of H.8 antigen was excessive. Fractions from the QAE chromatography (last part of second peak) were loaded on the column and eluted with the gradient described above. The chromatogram is shown in Fig. 4. Several peaks containing material absorbing at 280 nm (major peak shown at 20 min is the putative protein I) eluted ahead of those fractions (arrows) containing the H.8 antigen. No 280-nm absorbance was associated with the H.8 containing fractions.

Amino Acid Analyses.   Amino acid composition of purified H.8 was determined after hydrolysis of the material in 6 M HCl. A remarkable feature of the amino acid composition was the high content of alanine, glycine, and proline (Table I). Together, those amino acids made up 50% of the total amino acid content. The other notable feature was the lack of aromatic and sulfur-containing amino acids.

Assessment of Purity of H.8 Antigen.   As mentioned, the H.8 antigen is known to have several unique staining properties. It is refractory to staining by Coomassie Brilliant Blue and stains poorly with silver. To detect potential LPS or protein contaminants, we subjected the fractions containing purified H.8 to SDS-PAGE and analyzed the gels by staining and by immunoelectroblotting. For comparison, we included gonococcal whole-cell samples. The results are shown in Fig. 5. In purified H.8 samples (containing ~7 µg, as determined by amino acid analysis), no other proteins or LPS were detectable in the Coomassie- or silver-stained gels. The immunoelectroblots were probed with mAb 10 and hyperimmune rabbit whole-cell antisera. No other antigens were detected by these techniques. Overloading with 70 µg of purified H.8 resulted in aberrant migration behavior of the H.8 antigen (data not shown); however, no other antigens were detected in the sample.

The unique amino acid composition, specifically the absence of cysteine, methionine, tyrosine, tryptophane, phenylalanine, and histidine was used as an additional criterion to determine H.8 antigen purity. Possible lipidic contami-

TABLE I

Amino Acid Composition of Gonococcal H.8 Antigen

Amino acid	Residues per mole*	Residues per molecule‡
Aspartic acid and asparagine	1.4	13
Glutamic acid and glutamine	2.7	24
Serine	2.8	25
Glycine	4.8	43
Histidine	—	—
Arginine	1.0	7
Threonine	1.6	14
Alanine	11.4	102
Proline	2.3	16
Tyrosine	—	—
Valine	1.0	9
Methionine	—	—
Cysteine	—	—
Isoleucine	0.7	6
Leucine	1.9	17
Phenylalanine	—	—
Lysine	1.4	13

* Based on 1.0 residue per mole arginine.
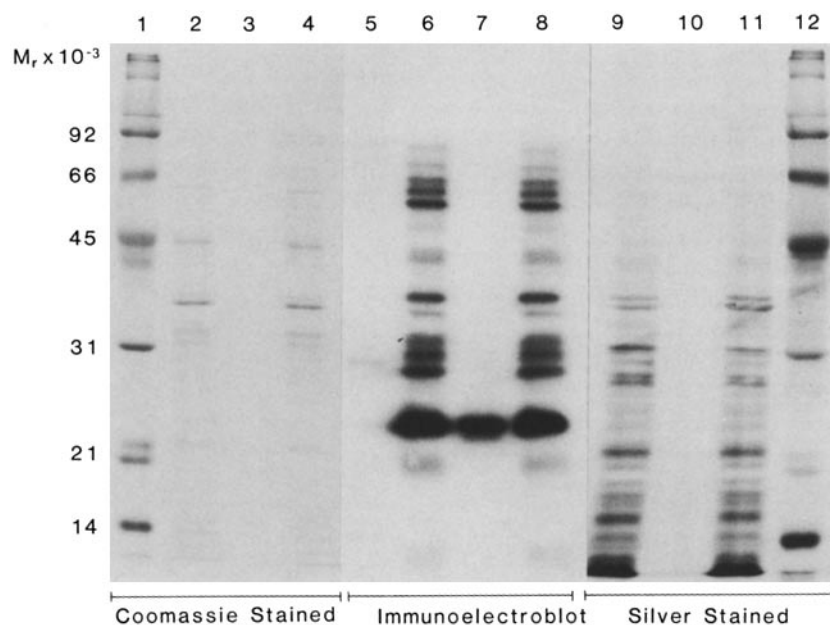‡ Residues per molecule (27,000 mol wt).

FIGURE 5. Comparison of purified H.8 with gonococcal whole-cell lysates by SDS-PAGE. Lanes *1, 5,* and *12,* M_r standards. Lanes *2, 4, 6, 8, 9,* and *11,* whole-cell gonococcal lysates. Lanes *3, 7,* and *10,* purified H.8. Immunoreactive components were identified using a gonococcal whole-cell antisera.

nants were monitored by gas/liquid chromatography, and ascertaining whether or not the LPS-associated fatty acids (18) 3-OH-14:0, 3-OH-12:0, and 12:0 and the phospholipid-associated fatty acids 16:0 and 16:1, were present. Methyl ester
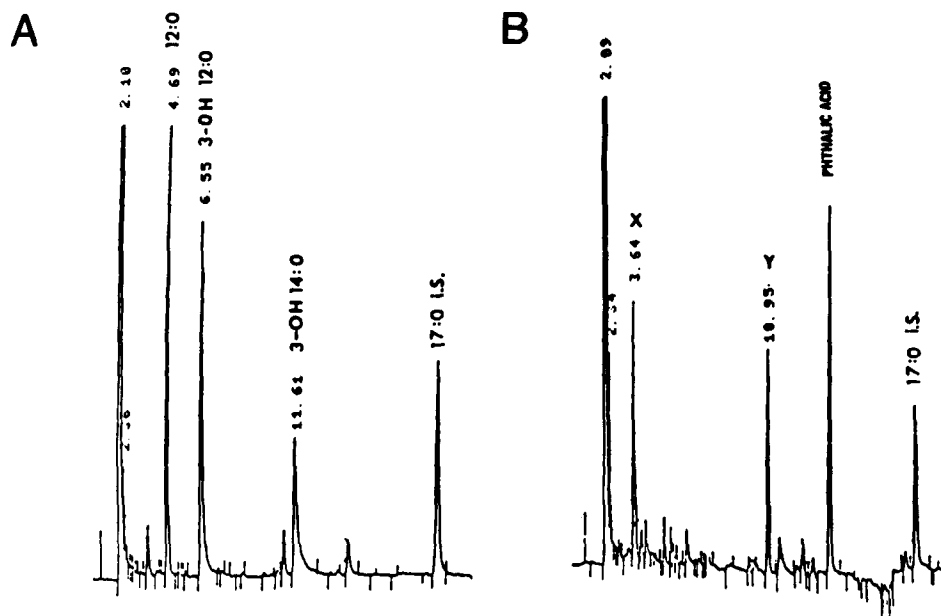
FIGURE 6.   Gas/liquid chromatography of gonococcal components liberated by methanolysis. (A) Fatty acid methyl ester released from purified gonococcal LPS. (B) Components X and Y released from purified H.8. Heptadecanoic acid methyl ester (17:0 i.s.) was used as an internal standard.

derivatives of those contaminants were detected in amounts of <1%, based on the protein content. On the other hand, two previously unidentified components with R_f values of 0.10 (X) and 0.29 (Y), relative to methylheptadecanoate, were found in purified H.8 samples after methanolysis. In Fig. 6, the two components detected in purified H.8 are compared to the fatty acid methyl esters released from purified gonococcal LPS. The two H.8-specific components could also be detected in whole-cell methyl ester derivatives, with a detector response comparable to the gonococcal 3-OH-14:0 (used as a marker for LPS) fatty acid content. Pthalic acid, an organic solvent–derived contaminant, was detected in purified H.8 samples.

Discussion

We have described a method for extraction and purification of the gonococcal H.8 antigen. Using a modification of an LPS extraction procedure, we were able to remove most of the H.8 from the bacteria. After extracting the H.8 with phenol/chloroform/petroleum ether, coextracted LPS was removed by aqueous precipitation. The phenol was then removed from the H.8 crude extract by gel filtration. Contaminating gonococcal protein (putative protein I) was removed by chromatofocusing and anion-exchange chromatography. In both purification steps, rather vigorous elution conditions such as pH 5.0–3.5 or high salt concentration (0.5 M NaCl) were necessary to recover the antigen. This could have been due to nonspecific (hydrophobic) interaction of the antigen with the column matrix. In the last purification step, based on reversed-phase chromatography, the molecule was retained on the column for a prolonged period. The retention

time was not in accordance with the theoretical value predictable from the amino acid composition (19) of purified H.8. The behavior of the antigen in these chromatography steps was consistent with the possibility that prosthetic groups such as lipids were associated with the protein constituent.

Results of preliminary analyses of the H.8 antigen revealed a number of interesting features; to a large measure, these results were consistent with the remarkable characteristics ascribed to H.8 antigen. The absence of aromatic amino acids was confirmed with the absence of absorbance at 280 nm. The hydrophobic nature of H.8 correlates with its mainly nonpolar amino acid content. The absence of sulfur-containing amino acid was predictable based on the lack of incorporation of [^{35}S]methionine (2) into the H.8 antigen, as well as its resistance to cleavage by CNBr, indicating the lack of cleavable methionine (W. Strittmatter, unpublished observation). The amino acid composition was remarkable in that the protein component was extremely rich in alanine and proline compared to previously characterized (20) gram-negative membrane proteins.

Purified H.8 was shown to be free of 16:1, and 16:0, as well as 3-OH-14:0, 3-OH-12:0, and 12:0 fatty acids, indicating there was no contamination with gonococcal phospholipids or LPS. However, two lipid components not previously identified or described in the gonococcous were detected in the whole-cell derivatives and enriched in the purified H.8 samples. Gas/liquid chromatography analysis data indicate that the smaller component has an R_f value comparable to a carbon chain with C10 or C11, while the second component had an R_f value comparable to 2-OH-14:0 fatty acid. The occurrence of unknown components described, as cyclopropane fatty acids, has been reported for Neisseriaceae (21). Further identification will require mass spectrometry–assisted gas/liquid chromatography to confirm the identity of those two components. It should also be noted that both molecules are liberated from H.8 upon transesterification in methanol/HCL, so artifacts arising from derivatization have to be taken into consideration.

It is appropriate to consider the relatedness of H.8 antigen enterobacterial lipoprotein (22, 23). Both H.8 antigen and lipoprotein lack phenylalanine and tryptophan and have similar Coomassie staining properties. However there was no uptake of [^{14}C]glycerol into the H.8 antigen (2), which means that the substituted glycerol anchor probably is not present. Although we were unable to detect cysteine by amino acid analysis, recently, incorporation of [^{35}S]cysteine into the H.8 has been demonstrated (P. Hitchcock, unpublished observation). Cannon and coworkers[3] have analyzed the predicted amino acid sequence of the H.8 antigen, based on DNA sequence of the cloned H.8 gene from meningococcal strain FAM 18, and have found the leader sequence contains a lipoprotein leader consensus sequence and an NH_3-terminal cysteine. It is possible that the lipid constituent may be amide-bound to the NH_3-terminal cysteine. It should be noted that there is no DNA homology between the lipoprotein gene and the gonococcal genome (H. Nikaido, Univ. California, Berkeley, personal communication). Whether H.8 might be functionally analogous to lipoprotein (to anchor the outer membrane to the peptidoglycan layer) is also unclear, since the

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[3] J. G. Cannon. 1986. Genetics of H.8 Antigen. Fifth International Conference on Pathogenic Neisseria. Amsterdam, The Netherlands.

extensive outer membrane blebbing normally observed in the gonococcus and the meninogococcus is similar to that seen in an *Escherchia coli* lipoprotein-deficient mutant (24).

A number of important questions remain to be answered regarding the purified H.8 material. First, we do not understand the chemical nature of the lipid constituents, and as a consequence, the quantitative relationship between the protein and lipid constituents is unknown. The epitope recognized by mAb 10 is lost upon protease treatment; however we do not know if the peptide alone or in combination with additional constituents make up the epitope, or if perhaps the peptide is conformationally required for recognition of the nonprotein constituent. We have not completely answered whether or not any carbohydrate is associated with the H.8 antigen, however preliminary radiolabeling experiments using [^{14}C]glucose and [^{14}C]acetate in the presence or absence of an additional source of unlabeled carbohydrate resulted in excellent labeling of most cellular constituents, but not the H.8 antigen (2). Finally, before this study, identification of the H.8 antigen was by antibody-dependent methods. This precluded reliable quantitation. As a consequence, we were not able to quantitatively evaluate the purification procedure with respect to enrichment of the antigen. This problem can now be overcome by either using purified H.8 as a reference in antibody-dependent assays, or alternatively, by using the unique lipidic component associated with H.8 as specific markers to quantitate the antigen. Work is underway to simplify and optimize the purification procedure by circumventing the anion exchange–based separation steps and substituting two sequential reversed-phase chromatographic steps.

Summary

We have presented a method for the extraction and isolation of the gonococcal H.8 antigen. There was no evidence of contamination by other gonococcal proteins, phospholipids, or LPS. The purified H.8 antigen was subjected to preliminary analysis and appeared to be a proteolipid consisting of both protein and lipid components. The amino acid composition was unusual; the peptide portion of the antigen was an alanine and proline–rich molecule that lacked aromatic and sulfur-containing amino acids. The overall amino acid composition is hydrophobic. A lipid constituent was also identified; it was made up of at least two lipid components, which were unique to the H.8 molecule. The chemical nature of the association of the protein and lipid is presently unknown, but it is clearly a tenacious one.

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