

ANALYSES OF GONOCOCCAL H8 ANTIGEN.

Surface Location, Inter- and Intrastrain Electrophoretic Heterogeneity, and Unusual Two-dimensional Electrophoretic Characteristics

BY PENNY J. HITCHCOCK,* S. F. HAYES,† L. W. MAYER,§ W. M. SHAFER,||
AND S. L. TESSIER*

*From the National Institutes of Health, National Institute of Allergy and Infectious Diseases,
*Laboratory of Microbial Structure and Function, †Laboratory of Pathobiology, Rocky
Mountain Laboratories, Hamilton, Montana 59840; the §Division of Bacterial Diseases,
Centers for Disease Control, Atlanta, Georgia 30333; and the ||Department of Microbiology
and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322*

The molecular nature of the gonococcal surface has been the subject of intensive study for the last two decades. Very recently (1, 2), a new outer membrane surface constituent, the H8 antigen, has been identified and cloned. This antigen is found (1) primarily among the pathogenic species of the genus *Neisseria*. Independently, studies have been in progress in this laboratory on a gonococcal surface constituent with unusual two-dimensional electrophoretic properties. Comparison of the specificities of the H8 monoclonal antibodies (mAb)¹ used in previous studies (1, 2) and mAb 10, used in these studies, revealed that these antibodies recognize epitopes on the same antigen.

From the initial mAb studies (1), it was clear that the H8 antigen on each strain shares at least one common surface-exposed epitope. Based on preliminary data (1), some degree of interstrain size heterogeneity was mentioned, and it was noted that no variation in H8 electrophoretic mobility was observed among opacity variants. It has been suggested (1, 2), based on the surface location, the common antigenic determinant, and the ubiquitous distribution among pathogenic *Neisseria*, that H8 might be a possible candidate for a subunit vaccine. However, given the ability of the gonococcus to present an ever-changing antigenic facade to its host, it is surprising that a common H8 epitope is exposed on the gonococcal surface. Gonorrhea is characterized by repeated, chronic, and asymptomatic infections, therefore it seems likely that either H8 is indeed antigenically heterogeneous or that the host's immune response to H8 antigen is not a deterrent to the establishment of infection.

Because H8 may be an important constituent in host-parasite interaction, we have reexamined the surface exposure of H8 using immunofluorescence, slide agglutination, and immune electron microscopy. We also examined in some detail the inter- and intrastrain electrophoretic heterogeneity of the H8 antigen;

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¹Abbreviations used in this paper: LPS, lipopolysaccharide; mAb, monoclonal antibody; P, piliation; P.I, *N. gonorrhoeae* protein I; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2018 LOCATION AND ELECTROPHORETIC CHARACTERISTICS OF H8 ANTIGEN

H8 variation among 21 strains and among pilus and opacity phenotypic variants within strains FA1090, JS3, and JS1 was examined. In addition, electrophoretic heterogeneity of H8 was analyzed in serum-resistant and neutrophil granule protein-resistant isogenic strains vis-a-vis sensitive strains. The behavior of H8 in a two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system was characterized.

Materials and Methods

Bacterial Strains. Strains and sources of *Neisseria gonorrhoeae* used in this study are listed in Table I.

Culture Conditions. Bacteria were grown on solid clear-typing medium (3, 4), at 37°C in a humidified 5% CO₂ atmosphere. Intrinsic labeling of gonococci with ¹⁴C-labeled amino acids (New England Nuclear, Boston, MA) was done using Wong-Shockley-Johnston's medium (5), both in the presence and absence of glucose as a carbon source. Radioactive amino acids (sp act 0.025 mCi) were added to the medium at the time of inoculation and during the log phase (*t* = 4 h) of growth.

Lipopolysaccharide (LPS). LPS was purified by hot phenol-water extraction of gonococci grown on clear-typing medium. Extraction procedures have been described earlier (6, 7).

TABLE I
Sources and Serogroups of Gonococcal Strains

Strain	Serogroup*	Source
JS1	II	J. Swanson [‡]
JS3	I	Swanson
JS5β	II	L. Mayer [§] /Swanson
JS5	II	Mayer/Swanson
UU1008	Unknown	Z. McGee [¶]
Rice 2	Unknown	McGee (P. Rice) [¶]
FA1090	Unknown	J. Cannon ^{**}
WS1	Unknown	W. Shafer ^{‡‡}
WS8	Unknown	Shafer
FA19	Unknown	Shafer
FA102	Unknown	Shafer
8658	I	T. Buchanan ^{§§}
5766	II	Buchanan
7122	I	Buchanan
8035	II	Buchanan
6611	II	Buchanan
7929	I	Buchanan
8660	III	Buchanan
7189	II	Mayer
7220	Unknown	Mayer
7221	Unknown	Mayer

* Serotyping was done by J. Knapp, University of Washington, Seattle, WA.

‡ NIH Rocky Mountain Laboratories.

§ Centers for Disease Control.

¶ University of Utah School of Medicine, Salt Lake City, UT.

¶ Harvard University Medical School, Boston, MA.

** University of North Carolina School of Medicine, Chapel Hill, NC.

‡‡ Emory University School of Medicine.

§§ University of Washington School of Medicine, Seattle, WA.

Polyclonal and Monoclonal Antibodies. The H8-specific mAb 10 was produced using the techniques described originally by Koeller and Milstein (8) with modifications by Chesebro et al. (9) and Barbour et al. (10). Polyclonal antiserum to whole gonococci was raised in rabbits (11), and was the gift of O. Barrera and J. Swanson (NIH Rocky Mountain Laboratories). Hyperimmune polyclonal rabbit antisera with specificities for pili and various protein II (P. II) were gifts of G. Schoolnik (Stanford University Medical School, Stanford, CA) and M. Blake (The Rockefeller University, New York), respectively. In some experiments, whole-cell antisera were adsorbed with either formalin-fixed whole cells or LPS (11, 12).

SDS-PAGE. Whole-cell lysates and proteinase K-digested lysates (13) were examined by the electrophoresis method of Laemmli (14), with modifications as described earlier (13). The second-dimension electrophoresis method has also been described previously (15). When proteinase K digestion preceded the second dimensional electrophoresis, an excised lane from the first dimension gel was placed in a sealed plastic bag with or without 1 mg of proteinase K (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) in 2% SDS lysing buffer and heated at 60°C for 60 min, placed on the second stacking gel, and electrophoresed (13). Low- M_r markers (Bio-Rad Laboratories, Richmond, CA) were used in some gels; the protein standards included phosphorylase B (92,250), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). M_r markers were iodinated by the chloramine-T method of Hunter and Greenwood (16).

Staining of Gels. Coomassie Brilliant Blue R250 (Fisher Scientific Co., Pittsburgh, PA), 0.2% wt/vol in 25% (vol/vol) isopropanol with 7% (vol/vol) acetic acid was used to stain proteins. The silver-stain method, which stains both proteins and LPS, has been described (13).

Electroblotting. Electroblotting from SDS-gel to nitrocellulose was performed with the buffer system of Towbin et al. (17) and Renart et al. (18) as modified by Barbour et al. (10). Electrophoretic transfer conditions were 15 V, <100 mA for 12 h at 18°C. In some cases, the phosphate system, as originally described by Burnette (19), was used for transfer, however, subsequent steps in the process were the same as above. After incubation of nitrocellulose-bound antigen with antisera, ^{125}I -radiolabeled protein A (New England Nuclear) was used to identify antibody (IgG)-antigen complexes (10).

Slide Agglutination. To determine if the H8 epitope was exposed on viable gonococci, slide agglutination assays were performed as described (20). Colonies from 18-h cultures, grown on clear-typing medium, were used.

Indirect Fluorescent Antibody Assay. 10 gonococcal strains and opacity and piliation phenotypic variants of three gonococci strains were assessed for the presence of H8 antigen. The method was essentially that of Barbour et al. (10) except that after air drying the bacterial suspension, the slides were heat fixed before methanol fixation.

Immune Electron Microscopy. The binding of mAb 10 to the surface of gonococci was demonstrated using fixed, unstained gonococci. Protein A-gold particles prepared by the method of Robinson et al. (21) were used to mark mAb 10 molecules.

Results

Surface Exposure of H8. It has been shown previously (1), using mAb adsorbed with viable gonococci, that an epitope on the H8 antigen was surface exposed. In this study, the question of surface exposure was addressed using four other techniques. mAb 10 was used in slide agglutination and indirect fluorescent antibody assays, and in immune electron microscopy. In addition, adsorption studies with polyclonal serum using intact, formalin-fixed gonococci were performed. Results from slide agglutination assays using viable 18-h cultures were positive, all gonococcal strains and phenotypes examined to date were agglutinable with mAb 10. Similar results were obtained using the indirect fluorescent antibody assay. The photograph in Fig. 1 demonstrates significant binding of

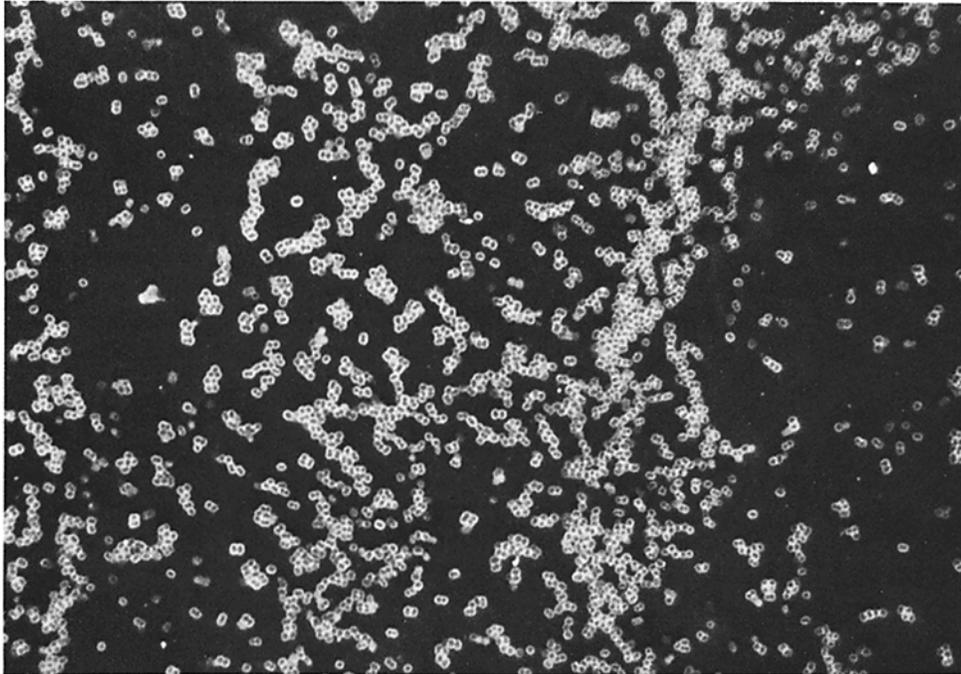


FIGURE 1. Indirect fluorescent antibody assay. mAb 10 was incubated with piliated (P^{++}) and nonpiliated (P^{-}) opacity variants from 10 different strains of gonococci. In all cases, the H8-specific mAb bound to the cell surface. This photomicrograph is representative of the fluorescent antibody binding pattern seen in all preparations (strain JS1, $P^{+}O^{-}$; 18-h culture).

mAb 10 to the gonococcal surface. No nonspecific fluorescence was observed in control preparations in which *Salmonella minnesota* R595 cells were used, or in which only a fluoresceinated anti-mouse Ig was used (data not shown). Using techniques designed to visualize the distribution of antigens on the outer membrane of gonococci by electron microscopy, the presence of H8 on the gonococcal outer membrane could be clearly demonstrated (Fig. 2). In this electron micrograph of an unstained diplococcus, large numbers of protein A-antibody-gold complexes were bound to the gonococcal surface. This photomicrograph is representative of the relative abundance and uniformity of H8-bound-mAb 10-protein A-gold complexes seen on all organisms on the grid. Some binding of these complexes to the grid was also observed.

H8 M_r Variation Compared with P.I and LPS M_r Variation. The variation in M_r of the H8 antigen was assessed in relation to the M_r of two other prominent surface antigens, (P.I) and LPS, in 13 strains. Results of these experiments are shown in Fig. 3, A, B, and C. In Fig. 3A, the P.I bands of 12 of the 13 strains can be seen (P.I of Rice 2, not shown, has the same M_r as JS1), the range of M_r is 37,500-34,000. The M_r heterogeneity in LPS can be seen in Fig. 3B. The M_r heterogeneity of the H8 can be seen in Fig. 3C. The range of M_r of H8 was 23,500-28,500. Although the H8 M_r of some strains were similar, e.g., JS1 and 8660, a detailed analysis of the electrophoretic mobilities of the three variables

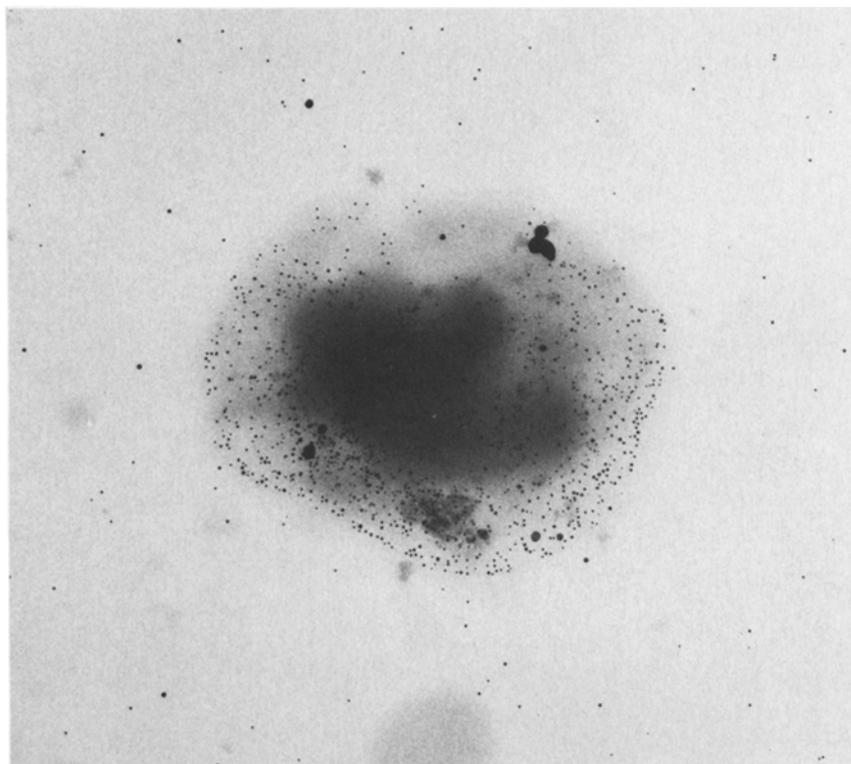


FIGURE 2. Immunoelectron micrograph of an unstained diplococcus incubated with mAb 10 and protein A-gold-labeled particles. The surface-exposed H8 antigen on the unstained organisms is discerned by the numerous gold particles.

revealed no correlation between the increase or decrease of the relative mobility of P.I, LPS, and H8.

Strain JS5 δ is a spontaneous nalidixic acid, rifampicin-resistant mutant derived from the JS5 β parent; concurrent with the acquisition of antibiotic resistance, a marked alteration of the M_r of the LPS was observed, however, the migration characteristics of H8 in the JS5 β and δ variants were identical (Fig. 3C). Hence, the LPS variation seen in JS5 β and δ seemed to be independent of H8 heterogeneity. To examine more carefully whether or not there might be any relationship between the M_r of LPS and H8, these characteristics were assessed in four isogenic organisms (WS1 and WS8 were constructed by W. Shafer). These variants differ in their resistance to killing by neutrophil granule proteins (W. Shafer, manuscript submitted for publication). The stained whole-cell lysates are shown in Fig. 4A. The parent F19 and the three related isogenic strains have the same P.I; this is shown in Coomassie Brilliant Blue-stained panel on the right. Note that H8 stained very poorly with the silver stain for LPS and proteins (and not at all with the stain that preferentially stains LPS [data not shown]). Examination of the M_r of the LPS of these strains (the LPS is bracketed in the center panel [it has been shown previously (22) that the M_r of gonococcal LPS is the same as that of the proteinase K-digested whole-cell lysate]) revealed that

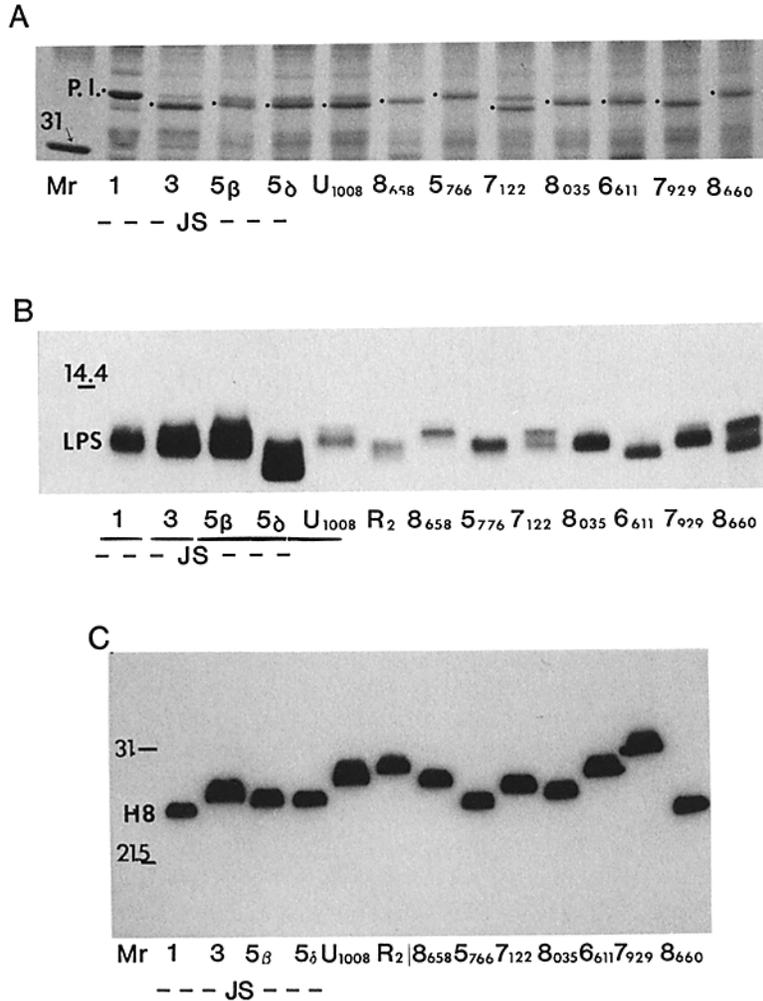


FIGURE 3. H8 M_r variation compared with P.I and LPS M_r variation. (A) The Coomassie-stained P.I are indicated by a black dot (●). The P.I M_r range from 37,000–34,000. Strain Rice 2 was not included in this gel; the P.I has the same M_r as JS1 (37,500). The gel has a 12.5% separating gel; ~20 μ g of protein was loaded for all samples stained by Coomassie Blue. (B) The silver-stained LPS patterns are shown in this gel. For strains JS1, JS3, and JS5, 2 μ g of purified LPS was loaded; in the other strains, the proteinase K-digested whole-cell lysate was used, an equivalent of 20 μ g of protein was loaded for each sample (18% separating gel). (C) An autoradiogram of whole-cell lysates immunoelectroblotted with mAb 10 is shown. The M_r of the H8 antigen ranges from 23,500–28,500 (12.5% separating gel).

the M_r of these LPS were related as follows: WS1 > FA19 > FA102, WS8. As can be seen in Fig. 4B, the M_r of H8 were slightly altered in the isogenic strains, the H8 M_r were related: WS8 > FA102, WS1 > FA19.

H8 M_r Variation in Serum Resistance. Given that H8 is present and exposed in the outer membrane, an obvious question arises as to its role in the well-documented serum sensitivity/serum resistance of *N. gonorrhoeae* (23). Originally, Hildebrant et al. (24) postulated that serum resistance was cotransformable with

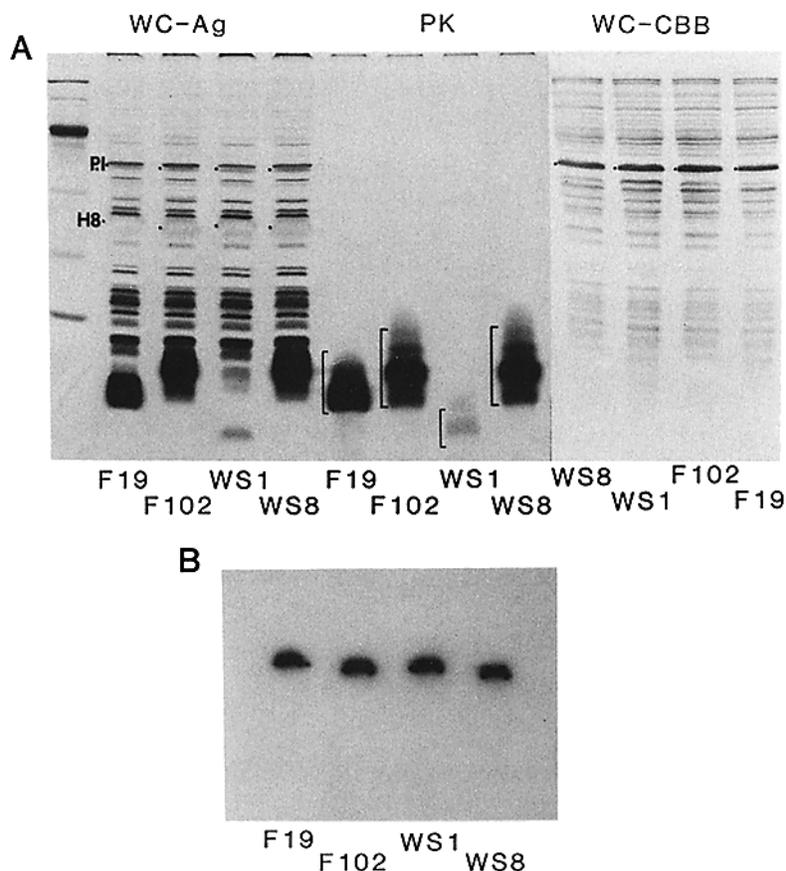


FIGURE 4. H8 M_r variation compared with LPS M_r variation. (A) Strains FA19, FA102, WS1, and WS8 whole-cell lysates were electrophoresed and stained with silver (WC-Ag) and with Coomassie Brilliant Blue (WC-CBB). P.I. is indicated with a black dot (●). The H8 antigen stains slightly (●). Juxtaposed are silver-stained proteinase K digests. The LPS is marked by brackets (]). We used an 18% separating gel, silver stained, or a 12.5% gel Coomassie stained. (B) Examination of an autoradiogram of immunoelectroblotted (mAb 10) whole-cell lysates reveals heterogeneity of the H8 M_r (12.5% separating gel).

changes in P.I phenotype. Subsequently, Cannon et al. (25) showed that the serum resistance characteristic(s) could be separated from changes in P.I phenotype. Using the strains originally constructed by Mayer, the H8 (and LPS) electrophoretic mobility was examined in the serum-sensitive recipient (strain 7189), and the intermediate (7220) and fully serum-resistant transformants (7221). The results of these experiments are shown in Fig. 5. Alteration in P.I M_r is apparent in 7221; the M_r of H8 has been altered in the intermediate and completely serum-resistant transformants (7220 and 7221) compared to the serum-sensitive recipient. The M_r of LPS may be altered, however, differential staining compromises this analysis (note that M_r of the various LPS shown in Fig. 6D are very reproducible).

H8 M_r Variation Compared to Variation in Piliation and Opacity Phenotype. The size and antigenic heterogeneity of opacity and pilus proteins in a given strain

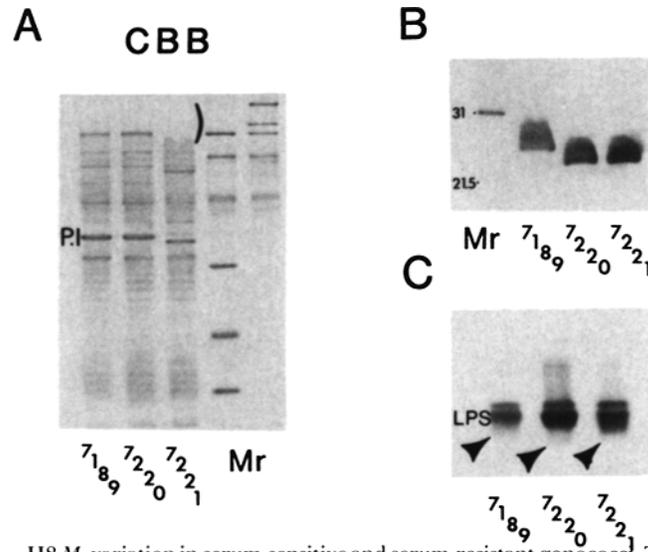


FIGURE 5. H8 M_r variation in serum-sensitive and serum-resistant gonococci. The Coomassie-stained P.I. are shown. The change in M_r is apparent: 7189, 7220 < 7221. An area (○) in the upper portion of the 7221 was refractory to Coomassie staining (12.5% separating gel). (B) The alteration in M_r of the H8 antigen in the transformants 7220 and 7221 is readily appreciated in this autoradiogram of immunoelectroblotted (mAb 10) whole-cell lysates (12.5% separating gel). (C) The silver-stained profiles of various LPS (2 μ g) are shown. A slight increase in M_r (▶) is apparent in the serum-resistant strains 7220 and 7221 compared to the serum-sensitive recipient (7189) (15.0% separating gel).

have been demonstrated by many investigators (reviewed in 26). The relationship between pilus subunit size and opacity proteins has also been scrutinized (27, 28). The relationship between variation in piliation and opacity phenotype and variation in H8 (and/or LPS phenotype) was examined using strains FA1090, JS3, and JS1. In strain FA1090, four opacity variants bearing P.II (P.II⁺) and a transparent (P.II⁻) variant were selected in both piliated colonies (with very sharp edges [P⁺⁺]) and nonpiliated (P⁻) colonies. The organisms were grown as described above. To insure the H8 and LPS were representative of a homogeneous population, the colonies that varied from the desired morphotype were removed from the agar before harvesting. As expected, the P.I were invariant among the colonial morphotypes, shown in Fig. 6A. The whole-cell lysates were probed for the presence of pilin after electroblotting using pilus-specific polyclonal antiserum; the autoradiogram is shown in Fig. 6B. A strong pilin signal was detected in the piliated but not in the nonpiliated variants. The P.II were visualized using the same technique; immunoelectroblots were probed with a P.II-specific polyclonal serum. In the gel shown in Fig. 6C, the whole-cell lysates were solubilized at 100°C and 37°C. By definition, the family of P.II is heat modifiable, however, this affect is not readily appreciated because P.II that are solubilized at lower temperatures are difficult to detect by this technique (11). The variation in P.II and the absence of a P.II signal in the P.II⁻ variant can, however, be appreciated. The LPS of these variants are shown in the silver-stained gel photographed in Fig. 6D. The M_r of all the LPS were identical. The

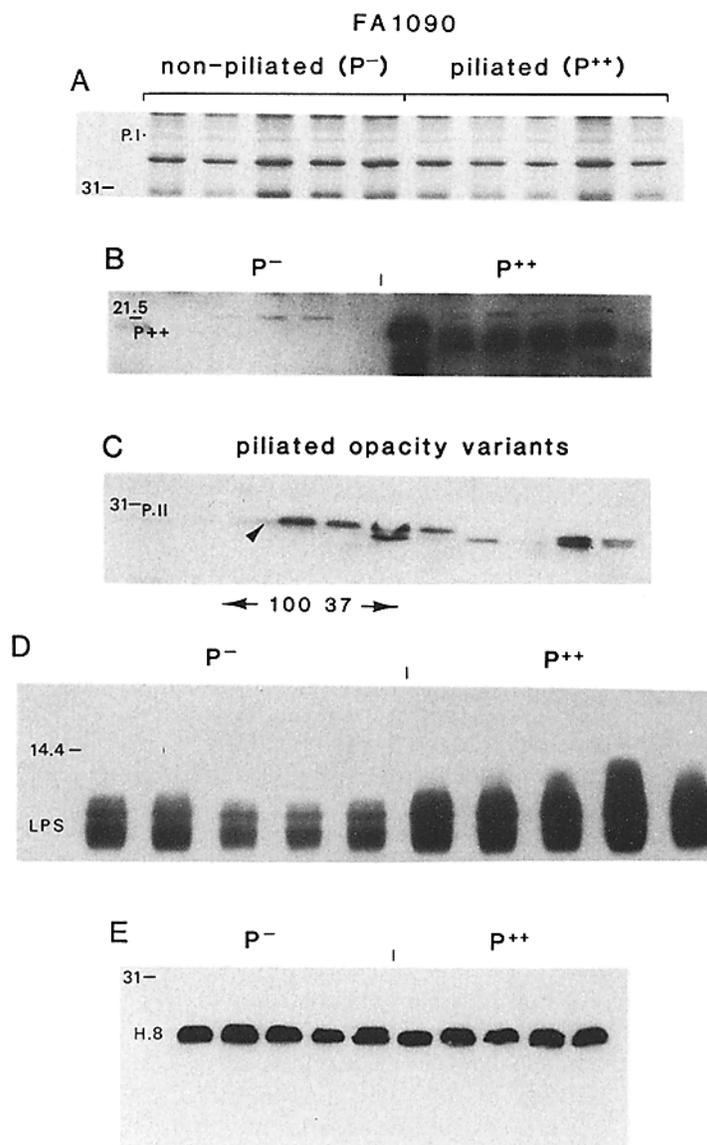


FIGURE 6. H8 M_r variation compared to variation in piliation and opacity phenotype. (A) The Coomassie-stained P.I are shown (12.5% separating gel). (B) Whole-cell lysates of P^- and P^{++} opacity variants were immunoelectroblotted with pilus antisera; a strong radioemitting signal can be seen in the five P^{++} variants (12.5% separating gel). (C) The whole-cell lysates of P^{++} opacity variants were immunoelectroblotted with P.II antisera. The lysates were solubilized at 100°C and 37°C. In the first two lanes are lysates of the P.II⁻ variant, some lateral smearing is noted (▶) from the adjacent P.II containing whole-cell lysate. A similar spectrum of P.II was seen in the P^- variants (data not shown) (12.5% separating gel). (D) The M_r of silver-stained LPS were identical for all variants of strain FA1090. The LPS of P^{++} variants stained darker, for unknown reasons (18% separating gel). (E) The M_r of H8 were demonstrated by immunoelectroblotting (mAb 10); all were identical (12.5% separating gel).

LPS preparations from the piliated variants stained darker in all preparations. The reasons for this are unknown, since the same amount of LPS (2 μ g) was added to each lane, and the LPS purity was virtually identical in all preparations (data not shown). The electrophoretic characteristics of H8 in these variants can be seen in Fig. 5E; like LPS, the H8 M_r of all phenotypes was identical. Similar results were obtained in strains JS3 and JS1, data not shown.

Unusual Electrophoretic Characteristics of H8 in One- or Two-Dimensional SDS-PAGE. The unusual mushroom cap-shaped band of H8 is similar to the shape of SDS-PAGE profiles of LPS in the whole-cell lysate, or to that of glycoproteins (22, 29). Since LPS migrates aberrantly in second-dimension gels (using Laemmli system in both dimensions), this technique was used (15) to analyze the H8 antigen. The whole-cell lysate of strain JS1 was electrophoresed by two-dimensional SDS-PAGE and immunoelectroblotted using rabbit hyperimmune sera elicited against homologous viable gonococci. An autoradiogram of this blot is shown in Fig. 7A. In addition to the typical LPS tail (labeled LPS in Fig. 7) another constituent of the whole-cell lysate migrated aberrantly in the second dimension. When the experiment was repeated using mAb 10 (Fig. 7B), this constituent proved to be the H8 antigen. In Fig. 7A, several discrete bands (bands 2–4) can be seen, in addition to the H8 tail. These bands are not recognized by the mAb in the one-dimensional whole-cell lysate shown to the right in Fig. 7B. The P.I of JS1 has M_r 37,500, which is very similar to that of band 1. To determine whether or not this higher M_r form was related to P.I, strain JS3 (P.I of 34,000 M_r) was first extrinsically labeled with 125 I (P.I has been shown [5] to be the most prominently labeled band in the 125 I-labeled whole-cell lysate), and used in a similar electrophoresis and immunoblotting experiment. A very small amount, equivalent to 5 μ g of protein, of whole-cell lysate was used so that the radioemitting signal from the 125 I-label would not obscure the mAb 10–antigen– 125 I–protein A complexes; because of this, only band 2 is shown; increased exposure time permits visualization of bands 3 and 4. From results of this experiment (Fig. 7C), it was apparent that band 2 was not associated with P.I, but more likely reflected one of three multimeric states of the H8 antigen. To determine whether or not these bands represented multimers, the experiment was repeated using two strains with H8 of quite different M_r ; JS3 (23,500) and 7929 (28,500). The M_r of the bands in JS3 were: 1, 23,500; 2, 43,600; 3, 86,000; and 4, > 150,000. In 7929, the M_r were: 1, 28,500; 2, 51,020; 3, > 100,000; and 4, >150,000. These values could be consistent with multimers of H8.

Association of H8 with LPS. To assess whether or not the two-dimensional migration characteristics of H8 were related to its association with LPS or an LPS-like constituent, sensitivity to proteinase K was assessed. In this experiment, the whole-cell lysate was electrophoresed in the first dimension, the lane was excised from the gel and incubated with proteinase K as described earlier (22). Subsequently, the second-dimension gel was immunoelectroblotted using whole-cell antisera (as in Fig. 7A). After proteinase K digestion, only the LPS tail was recognized by the antibodies (Fig. 8A). To determine whether or not the H8 antigen might be associated with LPS, an immunoelectroblot was probed with antisera that had been adsorbed with homologous LPS, shown in Fig. 8B. Antibodies to the LPS tail, but not to the H8 tail, were removed by adsorption

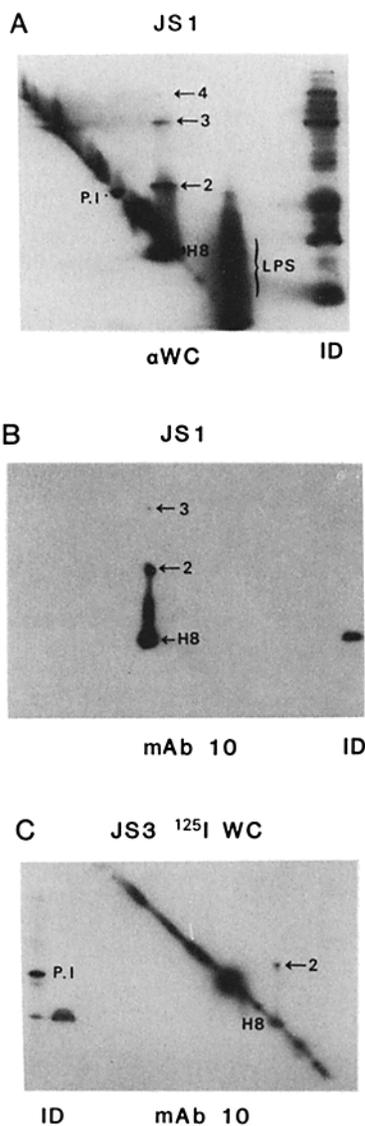


FIGURE 7. Autoradiograms of immunoelectroblotted two-dimensional polyacrylamide gels. Strains JS1 (A and B), and ^{125}I -labeled JS3 (C) P^-O^- whole-cell lysates were electrophoresed, transferred to nitrocellulose paper, and incubated with homologous whole-cell antisera (A) or mAb 10 (B and C). P.I., LPS, and H8 are designated. Bands representing multimeric forms of H8 are marked (2, 3, and 4). In each experiment, a control whole-cell lysate was electrophoresed in the first dimension (ID).

with LPS. In contrast, H8- and LPS-specific antibodies were both removed when antisera were adsorbed with formalin-fixed homologous whole gonococci (Fig. 8C). RNase-treated whole-cell lysates were also examined, since there is an RNA band that migrates in this area of the whole-cell lysate (P. Hitchcock, unpublished observation). RNase treatment destroyed the RNA bands, but had no effect on the two-dimensional mobility of H8 (data not shown).

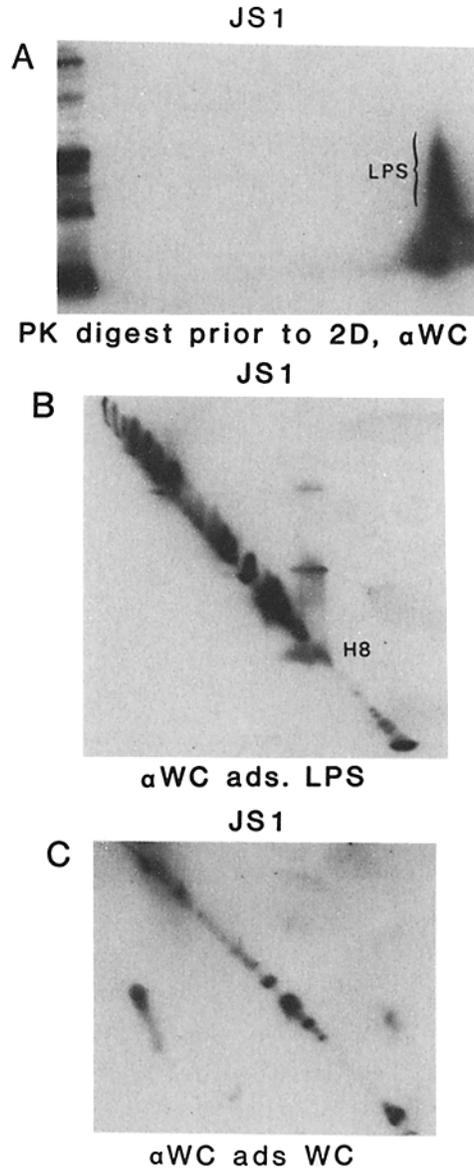


FIGURE 8. Autoradiograms of immunoelectroblotted two-dimension polyacrylamide gels. Strain JS1 P⁻O⁻ was electrophoresed, transferred, and incubated with homologous whole-cell antisera. In *A*, the first-dimension lane was excised from the gel and incubated with proteinase K before second-dimension electrophoresis. Only the LPS tail (}) was detected by the antisera. In *B* and *C*, the experimental parameters were the same as for Fig. 7*A*, however, the antisera was adsorbed with LPS (*B*) or whole cells (*C*). In *B*, the LPS tail is not seen, the H8 antigen (▷) is still recognized by LPS adsorbed sera. In *C*, the adsorption of sera with formalin-fixed whole cells removed antibodies to LPS and to H8.

Radiolabeling of H8 Antigen. Gonococci were grown in a defined liquid medium (5) in the presence of a mixture of ¹⁴C-labeled amino acids, as described above. Organisms were harvested and solubilized in 2% SDS-lysing buffer, and

electrophoresed in a two-dimensional SDS-polyacrylamide gel. An autoradiogram of the gel is shown in Fig. 9A. Two radioemitting spots, which have the same two-dimensional electrophoretic migration characteristics as the H8 antigen, are noted (dark arrowheads). As a control, labeled organisms were electrophoresed and immunoelectroblotted using mAb 10; the results of this experiment are shown in Fig. 9B. The ^{14}C -labeled spots comigrate with antigen(s) that is recognized by the H8-specific mAb.

Discussion

In this study, the surface location of the H8 antigen was confirmed by several methods; in addition, the immune electron microscopy results were suggestive of the presence of H8 in abundant quantities in the outer membrane. Based on the electrophoretic characteristics of the antigen, we confirmed that a considerable amount of interstrain heterogeneity was detected, however, the antigen had at least one epitope common to all strains examined thus far. Variation in H8 seemed to be independent of P.I and LPS variation. As had been reported previously (1), in strain FA1090, H8 appeared to be homogeneous; in addition, no H8 variation was seen among piliation and opacity variants of strains JS3 and JS1. The H8 antigen had an unusual banding pattern in one-dimensional gels, and migrated aberrantly in two-dimensional SDS-PAGE. The migration characteristics were similar to those of LPS; association with LPS or with a carbohydrate or lipid moiety could account for the unusual electrophoretic characteristic, however, based on adsorption studies, no evidence for H8-LPS association was found.

The H8 antigen is reported (1) to be refractory to staining with Coomassie

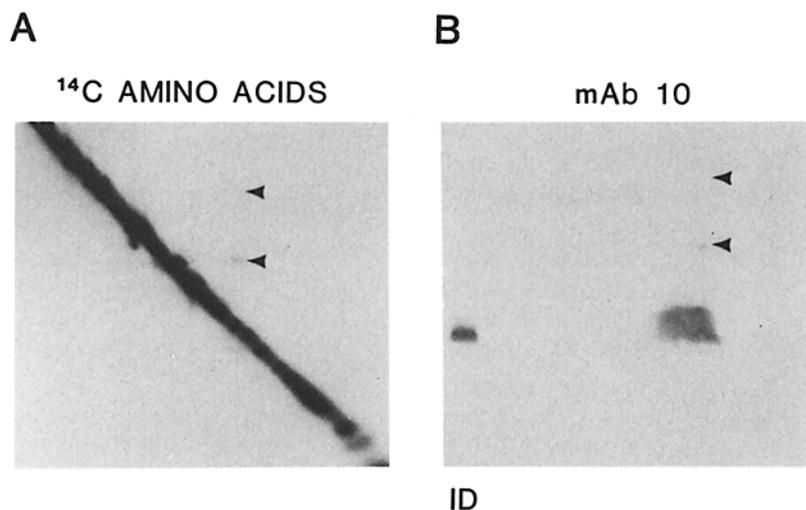


FIGURE 9. Autoradiograms of two-dimensional polyacrylamide gels of [^{14}C]amino acid-labeled gonococci. Strain JS3 was grown in Wong-Shockley-Johnston's medium in the presence of [^{14}C]amino acids. Whole-cell lysates were electrophoresed in two-dimensional SDS gels. In A, an autoradiogram of the gel is shown. Two radiolabeled spots are noted (▶); these have the same electrophoretic mobility as the H8 multimers. In B, the gel was immunoelectroblotted using the H8-specific mAb. The H8 antigen comigrates with the ^{14}C -labeled spots.

Brilliant Blue, and is only poorly stained with silver; these findings are consistent with the results of our study. Our attempts to radiolabel the antigen extrinsically with ^{125}I using several catalysts (chloramine-T, Iodogen, and Bolton-Hunter reagent) were unsuccessful (data not shown). We could not demonstrate incorporation of ^{32}P into H8 antigen when gonococci were grown in dephosphorylated complex medium. Success in intrinsically labeling H8 in gonococci grown in defined medium has also been limited. We have tried unsuccessfully to label with [^{35}S]methionine in methionine-free medium, [^{14}C]glucose in the presence and absence of cold glucose, [^{14}C]acetate, [^{14}C]glycerol, and *N*-[^{14}C]acetylglucosamine. A small amount of label was incorporated using ^{14}C -labeled amino acids in the presence or absence of cold glucose as a carbon source; this is shown in Fig. 9A. In all labeling experiments, H8 was synthesized in amounts readily detectable using mAb (Fig. 9B). The reasons for the difficulty in labeling this antigen are presently unknown. Hence, due to refractory staining and labeling properties, this surface antigen has gone unrecognized until the advent of immunoelectroblotting and mAb technology.

The structural significance of the interstrain electrophoretic heterogeneity of H8 is unknown. It is important to point out that the M_r of H8 might not always reflect biologically important biochemical differences. As with any other technique whereby two molecules are compared, identical migration characteristics do not imply identical molecules, however, electrophoretic heterogeneity in SDS-PAGE probably reflects molecular heterogeneity.

It had been reported (1) that H8 was heat modifiable in strain 1090. However, we were unable to demonstrate heat modifiability of H8. Reactivity of mAb 10 with a 30,000 M_r band was observed occasionally, however, we were unable to determine the experimental parameter that affected the reactivity of mAb 10 with this slow-migrating antigen. The M_r of the 30,000 band was invariant irrespective of H8 M_r ; the 30,000 M_r band did not correspond to any of the molecular species observed in two-dimensional electrophoresis.

The unusual staining and electrophoretic properties of this antigen bear additional comment; both may be indicative of the antigen being a complex one. Bacterial lipoprotein, LPS, and carbohydrate capsule stain poorly or not at all by either Coomassie (lipoprotein, LPS) or silver stain (carbohydrate capsule). And LPS has been shown to have unusual electrophoretic characteristics when analyzed by the two-dimensional gel system (14). This behavior is also seen in lipid A and the bacterial carbohydrate capsule of Contagious Equine Metritis Organism (P. Hitchcock, unpublished observation). The possibility that tightly-associated lipid or carbohydrate may be responsible for these electrophoretic properties of H8 is being investigated.

The biological function of H8 is presently unknown. The observation (1) that it is present in the outer membrane, perhaps in large quantities, and that it is found predominantly in the pathogenic *Neisseria* species implies that it may play a role in bacterial-host interactions. Analyses of sera from patients recovering from disseminated gonococcal and meningococcal infection revealed the presence of H8-specific antibodies, the inference is that H8 is present *in vivo*, at least on disseminated isolates (30).

The possibility that H8 may be a factor to consider in serum resistance is

suggested from the analyses of the 7189, 7220, and 7221 strains. In both of the serum-resistant transformants (7220 and 7221), the H8 phenotype was similarly altered. Our interpretation of these results is that neither H8 nor P.I transformation alone is sufficient for complete serum resistance, but that phenotypic alteration of both is required. This might be due to surface synergism between P.I and H8, or to separate populations of organisms, or to some additional unknown characteristic that is cotransformed with P.I and H8. The question of whether H8 phenotype is important in resistance to killing by neutrophils is raised in the H8 alterations observed in isogenic strains related to FA19 (i.e., FA102, WS1, and WS8). These organisms also differed in serum resistance (all, except WS1, are serum resistant, data not shown). Generation of H8 transformants with altered serum sensitivity, or with altered resistance to neutrophil proteins, as well as isolation and characterization of the H8 antigen from a number of serum-resistant and -sensitive strains may help answer these questions.

The biological significance of the interstrain electrophoretic heterogeneity of H8 is unknown. As a reflection of antigenic homogeneity, both polyclonal sera and mAb 10 and H8 crossreact broadly with H8 antigen in all gonococcal strains examined thus far. So far as intrastain homogeneity is concerned, among opacity and piliation variants of strains FA1090, JS3, and JS1, H8 appears to be invariant, as measured by these methods. Intrastain H8 variation is presently being examined in other strains. Whether the intrastain homogeneity demonstrated *in vitro* reflects the homogeneity of H8 *in vivo* is unknown. Although P.III also appears to be a common surface antigen (31), it seems paradoxical that an organism that relies on antigenic variation to insure its survival against the host's defense system (32) might have a stable, surface-exposed immunogenic molecule abundantly distributed on its outer membrane. Perhaps antibodies specific for H8 (and for LPS) are not effective in preventing or eliminating local infection. In this vein, we are also interested in exploring whether or not the H8 antigen may be involved in receptor-ligand interactions seemingly required in attachment of parasite to host. Based on electron microscopy studies, the amount of surface-exposed H8 seems to be considerable. We speculate that antibodies bound to H8 might interfere sterically with the interaction of gonococcal surface constituents and host-cell receptors. Further studies using an antiidiotypic mAb 10 may prove helpful in determining the function of H8 vis-a-vis host-cell interactions.

Summary

The H8 protein is a surface-exposed antigen that is found, among members of the *Neisseria* genus, primarily on pathogenic species (1). In this study, the surface exposure of H8 was reassessed by four techniques. Results of slide agglutination, indirect fluorescent antibody binding, adsorption of sera with whole gonococci, and immune electron microscopy all confirmed the presence of H8 in the outer membrane. The degree to which protein A-gold-labeled monoclonal antibodies bound to H8 was marked, and suggested that this antigen was present in abundant amounts in the outer membrane. Also in this study, the electrophoretic heterogeneity of this common surface antigen was examined. Because H8 stains poorly, electrophoretic mobility was assessed using polyclonal antibodies and a monoclonal antibody that recognizes a common H8 epitope.

H8 was analyzed with respect to protein I, lipopolysaccharide (LPS), and pilus and opacity phenotypic variation; results confirmed that heterogeneity of M_r was the rule among strains (21 were examined), however, the variability in M_r was independent of protein I or LPS M_r . In one strain (FA1090), the heterogeneity of H8 was examined among 10 piliation/opacity variants; the H8 (and LPS) M_r was identical in all variants; similar data were generated in strains JS3 and JS1. The electrophoretic mobility of H8 was altered in serum-resistant and neutrophil enzyme-resistant gonococci compared to the sensitive gonococci. Some of the unusual electrophoretic migration characteristics of the antigen were also examined. H8 formed a unique mushroom-shaped band in one-dimensional gels; in a two-dimensional electrophoresis system, the antigen migrated aberrantly, very similarly to LPS. Also seen in the two-dimensional electrophoresis profile were multimers of the H8 antigen; in strain JS3 (M_r 23,500), these migrated at 43,600, 86,000, and >150,000. In other strains, the M_r of the multimers differed depending upon the M_r of the monomer. The two-dimensional migration characteristics (as measured by antigenicity) were completely destroyed by proteinase K digestion. Activity of H8 polyclonal antibodies to the antigens in two-dimensional gels was completely removed by adsorption of formalin-fixed whole cells, but was not affected by adsorption with LPS. These electrophoretic characteristics may reflect the close association of some nonprotein constituent, perhaps lipid or carbohydrate or both.

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2034 LOCATION AND ELECTROPHORETIC CHARACTERISTICS OF H8 ANTIGEN

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