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Purification and Antigenic Relatedness of Proteins II of *Neisseria gonorrhoeae*

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Gonococcal proteins II from three strains were purified by chromatofocusing, and antisera was raised against them. These antisera were examined by immunoblotting to explore the antigenic relatedness of proteins II of seven different strains. The strongest reactions of the antisera were with the homologous proteins II. The antiserum against the proteins II of one strain also reacted with the proteins II present in all of the heterologous strains, whereas the antisera against the proteins II of two other strains showed little cross-reactivity with heterologous proteins II. Monoclonal antibodies produced against the three proteins II of strain F62 were specific for homologous proteins II and recognized epitopes unique to each individual protein II. These studies confirm the extensive intra- and interstrain variability in the antigenic structure of these proteins.

The antigenic structure of the cell surface of *Neisseria gonorrhoeae* varies among strains due to the presence of antigenically distinct forms of different outer membrane components including protein I (9), lipopolysaccharide (1), pili (15), and proteins II (P.IIs) (3–5, 19). P.IIs are outer membrane proteins that can be identified by their surface exposure and heat modifiability (18). They are most frequently found in colonies or populations of gonococci that express the opaque (O) phenotype; however, they may also be expressed in transparent (T) gonococci, an example being the leukocyte association factor described by Swanson et al. (20).

All P.IIs studied thus far appear to be structurally related when analyzed by tryptic peptide mapping (17). However, minor differences have been observed for P.IIs that have different apparent molecular masses (17). Indeed, only one half of the tryptic peptides observed appear to be common to each of the P.IIs analyzed. Moreover, variable regions of P.II structure appear to be located on surface-exposed portions of the protein (7), thereby possibly providing a mechanism for antigenic diversity.

We report here a method for the purification of P.IIs by chromatofocusing. Using these purified P.IIs, we prepared polyclonal monospecific and monoclonal anti-P.II antibodies, and used these antibodies to assess the antigenic relatedness of the P.IIs present in seven different strains of *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacteria and media. *N. gonorrhoeae* strains were provided as follows: F62 from T. M. Buchanan, University of Washington, Seattle, and 7122, 7189, 7502, 17408, and 17275 from J. S. Knapp, Neisseria Reference Laboratory, University of Washington, Seattle. Nonpiliated opaque (O) and transparent (T) gonococci were maintained by serial, selective subculture on clear typing medium (16).

Outer membrane isolation. Outer membranes were isolated from cells harvested from plates and suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5, and ruptured in a Tekmar X-Press (Biox, Järfälla, Sweden) at −35°C. The frozen lysate from three passes through the X-Press at 1.5×10^5 to 2.0×10^5 Newtons per m^2 was thawed and incubated at 37°C for 10 min in the presence of 35 μg of DNase (Sigma Type V from bovine spleen), 35 μg of RNase (Sigma Type II-A from bovine pancreas) per ml and 1 mM $MgCl_2$. The suspension was sonicated for 30 s and then centrifuged at 10,000 × *g* for 10 min at 4°C to remove nonruptured cells. The supernatant was centrifuged at 48,000 × *g* for 60 min at 4°C. The resulting pellet was suspended to about 10 mg of protein per ml in 10 mM HEPES, pH 7.4, containing 0.2% (wt/vol) sodium *N*-lauroyl sarcosinate (Sarkosyl) to solubilize selectively the inner membrane (21). After incubation for 10 min at 25°C, the suspension was centrifuged for 60 min at 48,000 × *g*. The pellet was washed once in 10 mM HEPES, pH 7.4, by recentrifugation. The pellet of enriched outer membranes was suspended in the same buffer and stored at −70°C. Protein concentrations were determined by the method of Lowry et al. (13).

Isolation of gonococcal P.II. A suspension of gonococcal outer membranes in HBS (10 mM HEPES–145 mM NaCl), pH 7.4, containing 10 mg of protein was centrifuged at 48,000 × *g* for 1 h at 4°C to obtain a membrane pellet. This pellet was suspended in 1.5% (wt/vol) sodium deoxycholate–10 mM EDTA–50 mM glycine, pH 9.0, to a concentration of 2.0 mg of protein per ml. The suspension was incubated for 1 h at 37°C and then at 4°C for 18 h. Insoluble material was pelleted by centrifugation at 48,000 × *g* for 1 h at 4°C. The supernatant containing deoxycholate-soluble gonococcal proteins was desalted to remove deoxycholate by passage over a column (1.5 by 8 cm) of Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 1.0% (wt/vol) Triton X-100–10 mM HEPES, pH 7.4. This replacement with a nonionic detergent was required since the polybuffer exchanger resin used in chromatofocusing is positively charged and binds anionic detergents. Fractions corresponding to the void volume were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the location of P.II. Fractions containing P.II were pooled and stored at 4°C before being chromatofocused.

Chromatofocusing. Chromatofocusing was performed at 4°C with reagents obtained from Pharmacia, Uppsala, Sweden. A Pharmacia K9/30 column (0.9 by 30 cm) was packed

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to a height of 25 cm with the polybuffer exchanger PBE 118. The column bed was equilibrated with 25 mM triethylamine-hydrochloride–1% (wt/vol) Triton X-100, pH 11.0. The elution buffer was Pharmalyte pH 8-10.5 (0.0078 mmol/pH unit per ml), pH 8.0, that contained 1% (wt/vol) Triton X-100. Before applying the sample, 5 ml of elution buffer was run into the column. The sample was then applied (typically 5 ml containing 5 mg of protein), and elution was begun with Pharmalyte–Triton X-100, pH 8.0. The flow rate was adjusted to 19 cm/h, and elution was continued until the pH of the eluent stabilized at 8.0.

The pH of each eluent fraction was measured, and the protein profile of each fraction was determined by SDS-PAGE. Fractions containing pure P.IIs were pooled and then concentrated to 2 ml with a Diaflo PM 10 ultrafiltration membrane in a model 12 ultrafiltration cell (Amicon, Lexington, Mass.). The volume was then adjusted to about 15 ml with 10 mM HEPES, pH 7.4, and the sample again concentrated to 2 ml. This procedure was repeated three more times. Ultrafiltration effectively removed most of the Triton X-100 and the ampholytes present in the Pharmalyte elution buffer. The final concentrated, purified P.II was analyzed again by SDS-PAGE and then stored at −70°C.

SDS-PAGE. Samples of outer membranes were solubilized by incubation at 100°C for 3 min in a solution of 2.5% (wt/vol) SDS–1.25% (vol/vol) 2-mercaptoethanol–12.5% (vol/vol) glycerol–0.001% (wt/vol) bromophenol blue–62.5 mM Tris-hydrochloride, pH 6.8. Samples containing 15 μg of outer membrane protein were resolved on 12.5% polyacrylamide slab gels (140 by 90 by 1.5 mm) with the buffer system of Laemmli (12). The polyacrylamide of both the separating gel and the 3% stacking gel was 2.6% cross-linked with *N,N'*,-methylene-*bis*-acrylamide. Electrophoresis was performed at 20 mA per gel while the tracking dye was within the stacking gel and at 25 mA per gel thereafter.

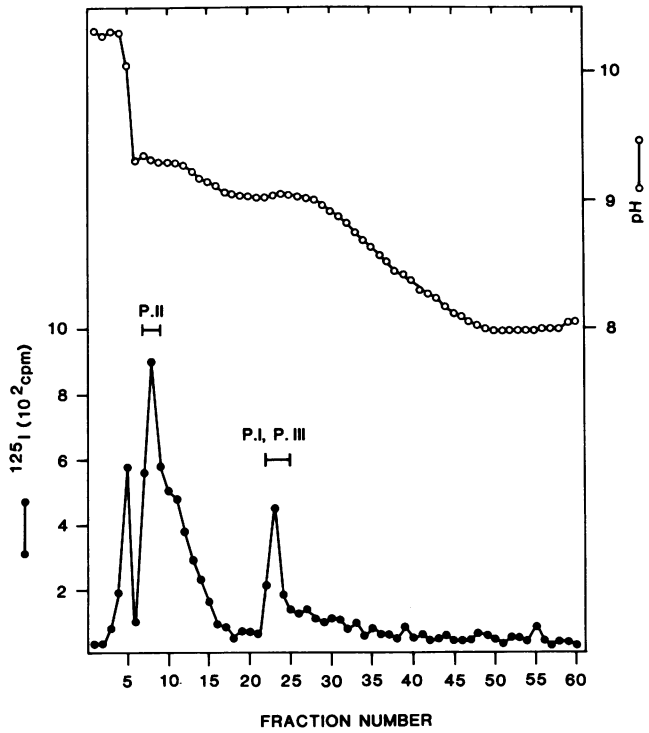

FIG. 1. Elution profile of deoxycholate-soluble outer membrane proteins of strain 7502 during chromatofocusing. For this experiment proteins were radiolabeled with ^{125}I by the method of Fraker and Speck (6) before extraction with deoxycholate. The fractions containing P.II are indicated with a bar. Similarly indicated is a peak that contained both P.I and P.III. The shoulder to the right of the P.II peak fractions represents a nonopacity-associated P.II that is present in this strain. No proteins were detected in the peak of radioactivity to the left of the P.II peak.

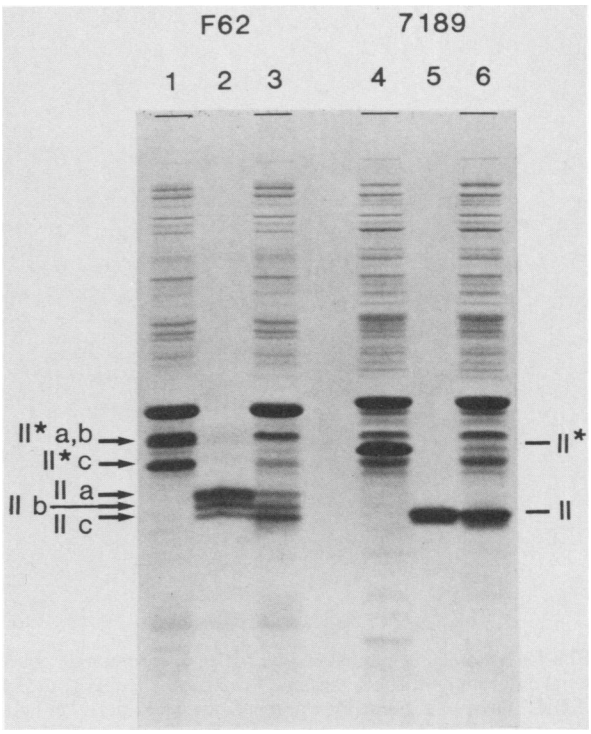

FIG. 2. Coomassie blue-stained polyacrylamide (12.5%) gel of outer membranes and purified P.IIs from strains F62 and 7189. Lanes: 1 and 4, outer membranes solubilized in SDS at 100°C; 3 and 6, outer membranes solubilized in SDS at 37°C for 1 h; 2 and 5, purified P.IIs solubilized at 37°C for 1 h. The positions of the 37°C-solubilized forms are indicated by II for strain 7189 and by IIa IIb, and IIc for F62. The heat-modified forms of P.II (100°C solubilized) are indicated by II*, II*a,b, and II*c.

Antigen-specific antibody detection. The details of the immunoblotting methods used have been described previously (2). Briefly, proteins resolved by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane and probed with monoclonal antibodies or mouse antisera raised against purified P.IIs. Bound immunoglobulin was detected by probing with ^{125}I-labeled protein A from *Staphylococcus aureus*. The nitrocellulose membrane was then used to expose Kodak X-Omat AR film.

Antiserum preparation. Mice were immunized intraperitoneally with 5 μg of purified P.II in HBS and emulsified in an equal volume of Freund complete adjuvant. Booster injections were administered on days 7 and 14 by intraperitoneal injection of 5 μg of P.II in HBS without adjuvant. Sera were collected on day 17 and titrated by immunoblot analysis.

Monoclonal antibodies. Murine monoclonal antibodies directed against P.II were produced by the method of Kohler and Milstein (11). Briefly, female BALB/c mice were immunized intraperitoneally with outer membranes in complete Freund adjuvant. Ten days later, the animals were given booster injections in the same manner and sacrificed three days postinjection. Splenocytes were mixed with Sp2/0-

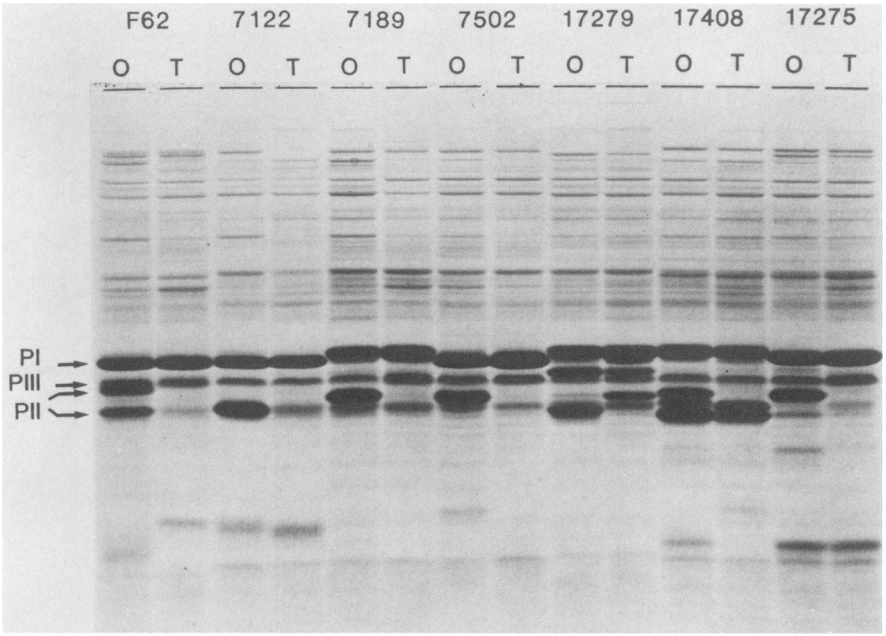

FIG. 3. Coomassie blue-stained 12.5% polyacrylamide gel of outer membranes from the opaque (O) and transparent (T) phenotypes of several different gonococcal strains. Strain and phenotype designations are given at the top of each lane. All samples were solubilized in SDS at 100°C. F62 is a laboratory reference strain. Strains 7189 and 7502 are isolates from patients with DGI. Strain 7122 has characteristics associated with DGI isolates. Strains 17279, 17408, and 17275 are genital isolates. The positions of proteins I, II, and III of strain F62(O) are indicated by arrows on the left.

Ag14 cells at ratios of 1:10 to 1:5 (splenocyte/Sp2/0). The mixtures were cosedimented and fused by brief treatment with polyethylene glycol 1500. After selection with HAT (hypoxanthine-aminopterin-thymidine) medium, cultures were screened against outer membranes by solid-phase enzyme-linked immunoassay. Antibody-producing cultures were screened by immunoblot analysis to detect those producing anti-P.II antibodies. Positive cultures were cloned in soft agar, picked, rescreened, and established as ascitic tumors in Pristane-primed BALB/c mice.

RESULTS

P.II purification. P.IIs were purified by using a chromatofocusing system that has maximum resolution in the pH range of 8 to 10.5 and takes advantage of their high isoelectric points (pIs) relative to those of other major outer membrane proteins (10). Figure 1 illustrates a typical fractionation profile. The pH gradient generated during elution under these conditions had a sharp decrease from pH 10.3 to 9.5, followed by a gradual decline to pH 8.0. Based on analysis by SDS-PAGE of the eluted fractions, all of the purified P.IIs eluted at a pH of about 9.3. This is consistent with the previously reported pIs of approximately 9.0 (10). Although at a pH of approximately 9.1 the eluted fractions contained both P.I and P.III, more than 90% of the P.I and P.III were retained on the column as predicted by their individual pIs.

The purity of P.IIs was assessed by SDS-PAGE (Fig. 2). F62(O) possessed three distinguishable P.IIs when the outer membranes were solubilized at 37°C. These proteins were designated as P.IIa, b, and c, and had apparent molecular masses of 27,000 daltons (27K), 26K, and 24K, respectively. When the outer membranes were solubilized at 100°C, P.IIa and b comigrated as a single band (P.II*a,b) having an apparent molecular mass of 31K. Similarly, P.IIc increased in apparent molecular mass to 29K and was designated P.II*c. The preparation of purified P.II from strain 7189 gave a single band with an apparent molecular mass of 24K when solubilized at 37°C and an apparent mass of about 30K when solubilized at 100°C.

Immunological characteristics of P.IIs. Antigenic cross-reactivity among P.IIs from different strains was evaluated with a panel of seven gonococcal isolates which included a laboratory reference strain, three isolates from genital infections, two blood isolates from patients with disseminated gonococcal infection (DGI), and a genital isolate with characteristics associated with DGI isolates. SDS-PAGE examination of the protein profiles of outer membranes purified from the O and T variants of each strain revealed a wide variation in the number and distribution of P.IIs (Fig. 3).

To assess the antigenic relationships among P.IIs, the panel of proteins shown in Fig. 3 was transferred to nitrocellulose membrane and probed with mouse antisera to purified P.IIs or with anti-P.II monoclonal antibodies. The most extensive cross-reaction was obtained with the antiserum raised against the three P.IIs of F62(O) (Fig. 4). In addition to reacting with the prominent P.IIs in the O and T variants of the different strains, this serum also reacted with proteins in the T variants that had not been identified as P.IIs by Coomassie blue staining. The presence of these P.IIs may reflect the small number of O organisms in populations of T gonococci due to a low frequency phenotype-switching phenomenon (3, 18). In contrast to the extensive cross-reactivity observed with the antiserum to P.IIs of F62, the antisera to the P.IIs of 7189 and 7502 gave more restricted cross-reactivity patterns.

To explore the antigenic structure of P.IIs with more precision, murine monoclonal antibodies were produced against the P.IIs of F62. After analysis of 17 P.II-reactive antibodies from two separate fusions only three distinguish-

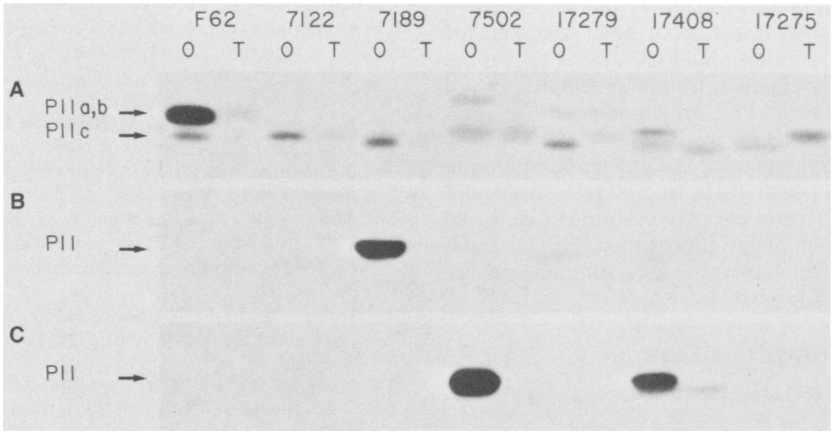

FIG. 4. Autoradiograms of sections of nitrocellulose membrane that contain proteins I, II, and III. For Panel A the gonococcal proteins were probed with mouse antisera to the chromatofocusing-purified P.IIs of strain F62. Panels B and C were obtained by using mouse antisera to the purified P.IIs of strain 7189 and strain 7502, respectively.

able specificities were identified. Each of the antibodies reacted with only one of the three P.IIs of F62, a, b, or c. No cross-reactions were observed with the P.IIs of the six other reference strains (Fig. 5).

DISCUSSION

The current interest in P.IIs derives from several characteristics associated with this group of proteins: (i) when present, P.IIs constitute a major proportion of the total outer membrane protein mass, often approaching that of the major outer membrane protein (P.I.); (ii) P.IIs express surface-exposed domains that can potentially interact with the host environment and possibly modulate gonococcal pathogenesis; and (iii) the expression of P.IIs exhibits phase variation in that a given strain is capable of displaying different P.II phenotypes depending on the methods of in vitro or in vivo culture. Together, these characteristics endow the gonococcus with the ability to alter dramatically its surface structure and may provide a mechanism to enhance survival in response to changes in the host environment such as those which occur during the menstrual cycle (8).

A unique characteristic of P.IIs is that they are basic proteins with pIs of about 9.0 (10). This is substantially higher than other outer membrane proteins such as P.I, which has a pI of about 8.0 (10). We took advantage of this difference in pIs for the purification of P.II by chromatofocusing. Using this method we were able to purify milligram quantities of P.IIs of various strains with relative ease.

The antigenic diversity of P.IIs has been demonstrated previously by using antisera to whole cells, purified P.IIs, and monoclonal antibodies (3–5, 19). In the present study a larger panel of more diverse strains (including DGI strains) was used to confirm and extend these observations. The broad cross-reactivity observed with polyclonal antisera is similar to that obtained by Swanson and Barrera (19) and provides additional evidence for the existence of common P.II epitopes. However, the antisera against the P.IIs from strains 7189 and 7502 reacted with only one or two P.IIs. Therefore, the hypothesis that the common antigenic domains of P.II are more immunogenic when the protein has been purified or denatured (19) may be true only for some P.IIs. In addition, when we tested antisera against whole cells of strains 7189 and 7502, we observed cross-reaction patterns nearly identical to those shown in Fig. 4 (data not shown). These data suggest that under the conditions of immunization that were used, only certain P.II epitopes are immunodominant and that this immunodominance is independent of the physical state of the intact protein. Alternatively, the differences between the results observed in the present study and those reported previously (19) may reflect variations in immunization schedules.

Two monoclonal antibodies specific for P.IIs have been identified by others (3, 14). One of the antibodies (1090-10.1) is specific for two of the six different P.IIs expressed by strain FA1090 (3). This antibody also reacts weakly or not at all with heterologous strains in a radioimmunoassay (14). However, the P.II profiles of the heterologous strains were not characterized, making it difficult to draw conclusions from those data regarding the antigenic relatedness of P.IIs.

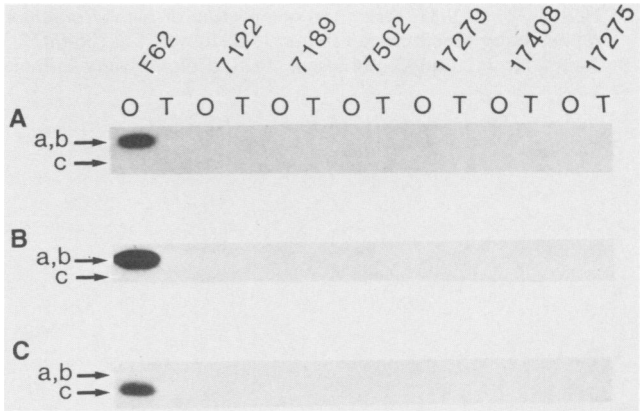

FIG. 5. Autoradiograms of sections of nitrocellulose membrane that bear the P.IIs of the seven different strains after reaction with (A) monoclonal antibody 53C4-1, specific for protein IIa; (B) monoclonal antibody 53A6-6, specific for protein IIb; and (C) monoclonal antibody 53A5-4, specific for protein IIc. The relative positions of proteins IIa, b, and c are indicted by arrows. To discriminate reactivity between P.IIa and P.IIb, immunoblotting experiments were performed with membranes solubilized in SDS at 37°C which allows IIa and IIb to be resolved (data not shown). However, antibody-antigen reactivity in such experiments was severely diminished, as previously reported (19).

The other monoclonal antibody (H138.2) is specific for a single P.II of strain FA1090 but has not been assessed for cross-reactivity with P.IIs in heterologous strains (3). In the present study, three monoclonal antibody specificities were identified for the P.IIs of strain F62. No cross-reactions were observed with heterologous P.IIs in six other strains. These data provide additional evidence for the existence of unique epitopes within the individual P.IIs of a single strain and leave little doubt that there is extensive strain-to-strain as well as intrastrain antigenic variation among P.IIs. Such variation could be important in governing interactions between the organism and the host.

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LITERATURE CITED

1. **Apicella, M. A.** 1976. Serogrouping of *Neisseria gonorrhoeae*: identification of four immunologically distinct acidic polysaccharides. J. Infect. Dis. **134**:377–383.
2. **Batteiger, B., W. J. Newhall V, and R. B. Jones.** 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods **55**:297–307.
3. **Black, W. J., R. S. Schwalbe, I. Nachamkin, and J. G. Cannon.** 1984. Characterization of *Neisseria gonorrhoeae* protein II phase variation by use of monoclonal antibodies. Infect. Immun. **45**:453–457.
4. **Blake, M. S., and E. C. Gotschlich.** 1984. Purification and partial characterization of the opacity-associated proteins of *Neisseria gonorrhoeae*. J. Exp. Med. **159**:452–462.
5. **Diaz, J.-L., and J. E. Heckels.** 1982. Antigenic variation of outer membrane protein II in colonial variants of *Neisseria gonorrhoeae* P9. J. Gen. Microbiol. **128**:585–591.
6. **Fraker, P. J., and J. C. Speck, Jr.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. **80**:849–857.
7. **Heckels, J. E.** 1981. Structural comparison of *Neisseria gonorrhoeae* outer membrane proteins. J. Bacteriol. **145**:736–742.
8. **James, J. F., and J. Swanson.** 1978. Color/opacity colonial variants of *Neisseria gonorrhoeae* and their relationship to the menstrual cycle, p. 338–343. *In* G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington D.C.
9. **Johnston, K. H., K. K. Holmes, and E. C. Gotschlich.** 1976. The serological classification of *Neisseria gonorrhoeae*. I. Isolation of the outer membrane complex responsible for serotypic specificity. J. Exp. Med. **143**:751–758.
10. **Jones, R. B., P. A. Jemison, W. J. Newhall V, and R. A. Haak.** 1980. Resolution of basic gonococcal outer membrane proteins by nonequilibrium pH gradient electrophoresis. Infect. Immun. **30**:773–780.
11. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
13. **Lowry, D. 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.
14. **Nachamkin, I., J. G. Cannon, and R. S. Mittler.** 1981. Monoclonal antibodies against *Neisseria gonorrhoeae*: production of antibodies directed against a strain-specific cell surface antigen. Infect. Immun. **32**:641–648.
15. **Rothbard, J. B., R. Fernandez, and G. K. Schoolnik.** 1984. Strain-specific and common epitopes of gonococcal pili. J. Exp. Med. **160**:208–221.
16. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19**:320–331.
17. **Swanson, J.** 1980. ^{125}I-labeled peptide mapping of some heat-modifiable proteins of the gonococcal outer membrane. Infect. Immun. **28**:54–64.
18. **Swanson, J.** 1982. Colony opacity and protein II compositions of gonococci. Infect. Immun. **37**:359–368.
19. **Swanson, J., and O. Barrera.** 1983. Immunological characteristics of gonococcal outer membrane protein II assessed by immunoprecipitation, immunoblotting, and coagglutination. J. Exp. Med. **157**:1405–1420.
20. **Swanson, J., E. Sparks, D. Young, and G. King.** 1975. Studies on gonococcus infection. X. Pili and leukocyte association factor as mediators of interactions between gonococci and eukaryotic cells in vitro. Infect. Immun. **11**:1352–1361.
21. **Walstad, D. L., L. F. Guymon, and P. F. Sparling.** 1977. Altered outer membrane protein in different colonial types of *Neisseria gonorrhoeae*. J. Bacteriol. **129**:1623–1627.