IMMUNOLOGICAL CHARACTERISTICS OF GONOCOCCAL OUTER MEMBRANE PROTEIN II ASSESSED BY IMMUNOPRECIPITATION, IMMUNOBLOTTING, AND COAGGLUTINATION

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Gonococci (Gc) vary widely in their outer membrane protein II (P.II) constitutions (1, 2). Some Gc exhibit no P.II; other Gc of the same strain have one or several different P.II. Each P.II can be present alone or in combination with other P.II. Several P.II subunit molecular weight forms have been described within individual strains and among different strains of Gc (1, 3, 4). All P.II have common characteristics including susceptibility to proteolysis, surface exposure, and "heat modifiability" (4). Furthermore, different P.II are structurally similar as deduced from 125I-peptide maps of electrophoretically separated P.II bands (5). But different P.II have differing apparent effects both on colony opacity (1, 4) and on the adherence of Gc to eucaryotic cells (3, 6–10). Structural differences among P.II are found by 125I-peptide mapping (5) especially when the proteins are radiolabeled in situ on intact Gc (11). The present study explores the antigenic and immunogenic relationships of P.II in general, with particular attention to the surface-exposed portions of these Gc outer membrane constituents.

Materials and Methods

Gc and Growth Conditions. These studies were done with strains JS1, JS3, JS5, and R10, whose colonial opacity variants were selected as described previously (4). These strains were selectively passaged multiple times on a clear medium whose composition has been described (12) and propagated at 35.5°C in a 5% CO2 atmosphere.

Antiserum against Whole P.II+ Gc. Adult rabbits were inoculated with once frozen (−70°C) and thawed portions of a stock suspension (100 Klett U, blue filter) of whole P.II+ Gc in phosphate-buffered saline (PBS) containing magnesium and calcium (4), according to the following schedule: 1st wk, two subcutaneous injections each with 0.5 ml; 2nd wk, three intravenous injections each with 0.5 ml; 2nd wk, three intravenous injections each with 0.3 ml and 0.4 ml portions, respectively; 3rd and 4th wk, intravenous injections of 0.5 ml each three times a week (2-d intervals). The rabbits were bled 1 wk after the final immunization and, after clotting and centrifugation, portions of the sera were frozen (−30°C) with added sodium azide (0.02%) and thawed for use.

Antiserum against Isolated P.II. Two antisera from rabbits immunized with specific, isolated P.II constituents were obtained from two colleagues. One of these sera (αP.IIj, R10) was the gift of Dr. Milan Blake, The Rockefeller University, New York. It was raised against a P.II that had been chromatographically purified in the presence of the detergent Zwittergent 3-14 (Calbiochem-Behring Corp., San Diego, CA). The other anti-P.II serum was a gift from Dr. 1

Abbreviations used in this paper: Gc, gonococci; PBS, phosphate-buffered saline; P.I, II and III, outer membrane proteins I, II, and III; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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Leonard Mayer, Rocky Mountain Laboratories. This serum (aP.IIfl, JS1) was from a rabbit immunized with P.II bands isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in which the proteins from outer membrane preparations of strain JS1 Gc had been resolved.

Absorption of Antisera. For immunoblotting and coagglutination studies, the rabbit antisera described above were absorbed with formalin-fixed, washed, whole P.II+ Gc of strain JS3 overnight at 4°C with end-over-end tumbling. These sera were diluted 1:3 with PBS, and 1.5 ml of this diluted serum was absorbed with 0.5 ml of an ~10% suspension of fixed, washed Gc.

Radioiodination of Gc. Whole organisms of desired strain and phenotype were 125I-labeled by the Iodogen reagent (Pierce Chemical Co., Rockford, IL) as described before (13).

Immunoprecipitation. The pellet of 125I-labeled Gc (equivalent to 1.5 ml of a suspension of ODs40 = 0.6) was suspended in 165 µl PBS, and 50 µl portions were placed in 1.5-ml microfuge tubes. 50 µl of rabbit serum was added to each and after mixing these were incubated in an ice bath for 15 min. The Gc were then washed thrice with 1 ml cold PBS, and the final pellet was suspended by vortex mixing in 210 µl of 1% Zwittergent 3-14 in PBS. This detergent suspension was incubated at 40°C for 1 h and was centrifuged (4 min, Microfuge B; Beckman Instruments, Inc., Palo Alto, CA). The supernatant (190 µl) was carefully collected and added to a tube containing a slurry of previously swollen Sepharose 4B-CL protein A (Sigma Chemical Co., St. Louis, MO) in PBS (2 mg in 40 µl per specimen). This mixture was incubated with end-over-end rotation for 15 min, and the sedimented pellet (30 s, Microfuge) was washed several times each with 1 ml of 2% Zwittergent 3-14 in PBS. 1 ml of absolute ethanol was added to the final washed pellet, and the tube allowed to stand for 15 min. The ethanol supernatant was aspirated and discarded after centrifugation, and the tube contents were evaporated to dryness in a boiling water bath. Solubilizing solution (4% SDS, 8% 2-mercaptoethanol, 0.125 M Tris, pH 6.8, and bromphenol blue) was added (25 µl), and the tubes were placed in a boiling water bath for 10 min. These specimens were separated in a 12.5% polyacrylamide slab gel (14); after electrophoresis, the gels were fixed in 25% 2-propanol, 7% acetic acid and exposed overnight at room temperature to x-ray film (XAR; Eastman Kodak Co., Rochester, N. Y.) both as wet gels wrapped in plastic and, later, after drying. This represents a minor modification of the method previously described (13) with increased amounts of both 125I-labeled organisms and antisera.

Coagglutination. A modification of the technique described by Kronvall (15) was used. Briefly, the absorbed antisera were diluted 1:10 with PBS containing 0.02% sodium azide and subsequent serial twofold dilutions were placed in microtiter plate wells (25 µl/well). Bacteria that had been previously fixed in 0.25% formaldehyde (16 h, room temperature), washed, and suspended to ~8 × 1010 Gc/ml were added as 25-µl volumes. The antiseraum-bacteria mixtures were incubated (37°C, 30 min) on a gyratory shaker and were washed, resuspended, and centrifuged again. To each well was added 50 µl of a suspension (10% suspension diluted 1:40) of Staphylococcus aureus Cowan I strain (ATCC 12598; American Type Culture Collection, Rockville, MD). The plates were incubated (37°C, 30 min) on the gyratory shaker, and coagglutination was read. Controls of normal rabbit serum, S. aureus Wood 46 strain (ATCC 10832), and serum-free mixtures were included. Wells were scored as positive (heavy clumping of Gc) or negative (no clumping or very slight clumping).

Immunoblotting. SDS-PAGE was carried out on unlabeled Gc that had been prepared as follows: 1.5 ml of a PBS suspension of Gc (OD400, 0.6) was centrifuged and the pellet suspended in 100 µl PBS. This suspension was frozen at -30°C and thawed when needed. To 10 µl of solubilizing solution (see Immunoprecipitation) was added 1 µl of the thawed Gc suspension that was boiled for 10 min before loading into the well of a 12.5% acrylamide gel and electrophoresis as described above. For each antisera to be tested, 10 such Gc lysates were used as a "battery" of test antigens and were electrophoresed in adjacent lanes along with a lysate of 125I-labeled organisms that served as controls in the subsequent electrophoretic transfer from gel to nitrocellulose sheets. For one study (as noted in Results), single nonpiliated colonies were picked up on filter paper fragments, immersed in 20 µl of solubilizing solution, boiled, and subjected to SDS-PAGE preparatory to immunoblotting. When the SDS-PAGE electrophoresis was completed, the slab gels were applied to nitrocellulose sheets (HAHY; Millipore Corp., Bedford, MA), and proteins were transferred in a Trans-Blot apparatus (Bio-Rad Laboratories,
Richmond, CA) at 60 V (0.2-0.23 A) for 3 h with the transfer buffer described by Towbin et al. (16). At the end of the transfer, the nitrocellulose sheet was incubated overnight on a rocking platform in a sealed bag with 2% bovine serum albumin. Dilution of the bovine albumin, immune rabbit sera, and 125I-labeled protein A from S. aureus, as well as all washing steps (except a terminal water wash), were done with the solution described by Renart et al. (17). Incubation with preimmune or immune sera was for 2 h in serum diluted 1:50, 1:100, or 1:1,000 as indicated in figure legends. The nitrocellulose sheets were washed (four changes, 30 min, and ~50 ml each) and then incubated with 125I-labeled protein A. Protein A was radioiodinated with chloramine T as published for a different protein (18). After incubation for 2 h on a rocking platform with 125I-protein A, the sheets were washed several times, finally washed with water, dried, and exposed to X-ray film (XAR; Eastman Kodak Co.) at room temperature for 20 h.

Results

General Comments. The two methods used most in this study—immunoprecipitation and immunoblotting—were chosen to provide somewhat different kinds of information about P.II antigens. The immunoprecipitation technique used here includes brief (15 min) incubation of whole, radioiodinated, P.II+ Gc with rabbit antiserum. This is designed to allow interactions between rabbit serum antibodies with surface-exposed portions of the Gc antigens. After washing to remove unbound antibodies, the Gc are lysed in Zwitterionic detergent, and the antibody plus 125I-labeled proteins are precipitated with Sepharose-protein A. Occurrence of a 125I-labeled Gc constituent in the immunoprecipitate is taken as evidence for interaction between surface-exposed antigen(s) of that protein constituent and antibody. Such protein or polypeptide antigens might have primary, secondary, or higher order structural determiners since they are in their "native" state (except for being radioiodinated).

Immunoblotting detects antigens in proteins that have been subjected to rigorous denaturation and "unfolding" by boiling in SDS; in this system, primary structure should be the major or sole determiner of antigenicity. Furthermore, antigens of P.II are available for reaction with antibodies by immunoblotting regardless of their being exposed or buried in the outer membranes of intact, whole Gc.

Several factors complicate rigorous interpretation of some of the results described below. First, there is a continual problem of having mixtures of P.II phenotypes in the sizable populations of Gc necessary for most of the techniques used. Previous studies have documented the high prevalence of P.II variants within progeny of a single colony (4). This is a particularly vexing problem in the preparation of immunogens, which requires large numbers of organisms; these large numbers practically assure the inclusion of more than one P.II phenotype in the Gc vaccines used to immunize rabbits. Care was taken to use only those cultures with few (~1%) opacity variants, but mixtures are unavoidable because variants occur with a prevalence of 1 per $10^5$-10^6 progeny upon passage of single colonies (4). Use of such mixtures could lead to antisera reactive against more than one "homologous" P.II and makes interpretation of minor apparent cross-reactivities difficult or impossible. It also presents difficulty in obtaining homogeneous populations of organisms used as antigens for immunoprecipitation or immunoblotting. A second problem concerns ambiguities inherent in arbitrary selection of autoradiography exposure times for both immunoprecipitation and immunoblotting. Additional variables such as differences in specific activities of 125I-labeled protein A preparations (with time and decay), different extents of radio-labeling of intact Gc for immunoblotting, etc., further complicate the interpretation
of the autoradiographs. However, these methods allow visualization of the gonococcal antigens with a specificity that is impossible with other techniques unless purified antigens are used.

In an attempt to minimize the variations inherent in the techniques chosen for this study, all the immunoprecipitation results presented (except those shown in Fig. 3) were carried out over a 3-wk period. Additional experiments were done over the course of a year and were consistent with the results presented, but only those done over the abbreviated period are depicted. Similarly, most immunoblotting results were obtained with a single batch of $^{125}\text{I}$-protein A over the course of a month’s study. Finally, all the figures shown for comparison of immunoprecipitation or immunoblotting results were obtained by identical factors of autoradiographic exposure, photographic reproduction, etc.

With the above-noted limitations in mind, the results described below demonstrate the following points: (a) As a group, P.II are immunogenic in rabbits both as isolated proteins and in situ on intact Gc. (b) Antisera raised by immunization of rabbits with whole P.II+ Gc contain IgG antibodies directed primarily toward surface-exposed portions of the homologous P.II, but examples of cross-reactivity or antigenic relatedness for different P.II of a single strain and among different strains can be found. These anti-whole P.II+ Gc rabbit sera exhibit similar reactivities by both immunoprecipitation and immunoblotting. (c) Antisera raised by immunization with isolated P.II material exhibit broad cross-reactivities among homologous and heterologous P.II by immunoblotting; the reactivities of these sera with different P.II constituents are much more restricted when they are used for immunoprecipitation.

**P.II+ Gc Studied.** P.II variants from four strains, JS1, JS5, R10, and JS3, were selected for study. These four strains include representatives of the three coagglutination serotypes (WI, WII, WIII) described by Danielsson and Sandström (19); they were assayed with monoclonal reagents as reported recently by Tam et al. (20). (Serotyping was kindly done by Dr. Joan Knapp, Seattle Public Health Hospital, Seattle, WA) The four strains have P.I whose subunit sizes are shown in Table I. For each strain, two or more distinct colony opacity variants are regularly found among progeny of a single P.II- transparent colony; their P.II are noted in Table I. An autoradiograph of $^{125}\text{I}$-labeled SDS-PAGE profiles of the P.II+ variants used for immunoprecipitation and immunoblotting is depicted in Fig. 1. Some P.II from different strains show identical apparent subunit sizes (e.g., JS1 P.IIa = JS3 P.IIa; JS1 P.IIβ = R10 P.II; R10 P.IIj = JS3 P.IIb). Strains JS5 and R10 each exhibit two P.II that are only slightly different in apparent subunit molecular weight. P.Ilaβ? variants of strain JS1 could be used since coexisting P.Ila and P.IIβ are easily distinguished.

**Immunoprecipitation with Antisera Raised against Whole P.II+ Gc.** In immunoprecipitation reactions, antisera raised against whole P.II+ Gc exhibited striking specificities toward different P.II. Fig. 2 depicts immunoprecipitation results for five P.II+ phenotypes (P.IIa+, P.IIb+, etc.) of strain JS3 Gc after their incubation with sera from rabbits immunized with whole Gc (strain JS3 or JS5) of the noted P.II+ phenotype (αJS3a+, etc.). Each antiserum yields an intense immunoprecipitation reaction with its respective, homologous P.II constituent (homologous mixtures of antiserum plus antigen indicated by X). Each antiserum also shows reduced but clearly visible (by the conditions used here for exposure of autoradiographs, etc.) “cross-reactions” with
Table I

P.I and P.II Characteristics of Gonococi Studied

| Strain (coagglutination type)* | P.I mol wt‡ | P.II phenotype | P.II mol wt§ | Colony opacity|| |
|-------------------------------|-------------|----------------|--------------|--------------|
| JS1 (VIII)                   | 36.5        | P.IIα*         | 29.0         | 3+           |
| JS1 (VIII)                   | 36.5        | P.IIβ*         | 30.5         | 1+           |
| JS3 (VIII)                   | 36.0        | P.III*         | 30.8         | 4+           |
| JS3 (VIII)                   | 36.0        | P.IIIg*        | 30.6         | 1+           |
| R10 (ND) ¶                   | 36.0        | P.III*         | 30.5         | 3+           |
| R10 (ND) ¶                   | 36.0        | P.IIIj*        | 30.2         | 1+           |
| JS3 (VIII)                   | 35.4        | P.IIa*         | 29.0         | 0            |
| JS3 (VIII)                   | 35.4        | P.IIb*         | 30.2         | 1+           |
| JS3 (VIII)                   | 35.4        | P.IIc*         | 30.5         | 4+           |
| JS3 (VIII)                   | 35.4        | P.IId*         | 31.0         | 2+           |
| JS3 (VIII)                   | 35.4        | P.IIe*         | 33.0         | 3+           |

* Coagglutination done on P.II⁻ derivatives of each strain; for strain JS3, coagglutination also done on P.IIc⁺ variants which were WI like the P.II⁻ organisms of that strain.
‡ By comparison with low molecular weight markers from Bio-Rad Laboratories.
§ Apparent subunit molecular weight given for specimens boiled for 10 min before SDS-PAGE.
|| Colony opacity scored on scale of 0-4+.
¶ Not done.

Fig. 1. SDS-PAGE autoradiograph of radioiodinated Gc used in this study. Gc from four strains, JS1, JS5, R10, and JS3, were selected for their having different P.II as described in the text. These organisms' major outer membrane proteins are seen in this portion of an autoradiogram of SDS-PAGE-separated, ¹²⁵I-labeled components after their transfer to nitrocellulose. Note the strain differences in apparent subunit sizes for protein I (I) as follows: JS1 > JS5 = R10 > JS3 (P.I mol wt given in Table I). Note also that all organisms have the same apparent mol wt P.III (III) constituents. Each of the P.II⁺ variants of strains JS5, R10, and JS3 has a single P.II (arrows) while the JS1 variant has two P.II. All these specimens were boiled for 10 min before SDS-PAGE, and under these conditions the various P.II had apparent subunit sizes noted in Table I.

heterologous P.II moieties (such as those seen for P.IIb, P.IIc, P.IId, and P.IIe with anti-JS3 P.IIa⁺ serum). P.IIa is not immunoprecipitated to a visible extent except by the homologous antiserum. Minor amounts of two P.II of strain JS3 (P.IIb and P.IIe) are found after immunoprecipitation with all the sera used; these include sera raised against organisms of a different strain (JS5). Antiserum against JS5 P.II⁺ organisms gives an intense immunoprecipitation reaction with strain JS3 P.IIb⁺; the reciprocal mixture (αJS3 P.IIb⁺ and JS5 P.II⁺ antigen) also showed cross-reactivity between the different strains' P.II. P.IIb is also recognized by serum raised against P.IIe⁺
Fig. 2. Immunoprecipitation of P.II by anti-whole P.II* Gc sera. Antisera were raised in rabbits immunized with whole Gc; each of the Gc immunogens contained organisms exhibiting a single P.II (P.IIa*, P.IIb*, etc.). Note that in this and subsequent figures, P.IIa* phenotype is given as a*, etc. The antisera used for immunoprecipitation are noted as αJS3 a*, αJS3 b*... αJS g*. The whole Gc used as antigens in immunoprecipitation are strain JS3 organisms; the autoradiographic profiles of SDS-PAGE-separated components of these organisms' whole cell lysates (WC) are shown (top panel). Note the constant occurrence and apparent size of proteins I (I) and III (III) in all these Gc, which differ one from another in their P.II constituents as indicated at the top of the panel. These five different P.II variants were radioiodinated and incubated with each antiserum, as shown. In each immunoprecipitation using the five P.II* antigens and the noted antiserum, the homologous combination is noted (X); also the position of P.I is indicated in each set of immunoprecipitates. Note the relatively minor amounts of proteins I and III present in these immunoprecipitates. Each antiserum immunoprecipitates its homologous P.II more extensively than heterologous P.II species; but minor amounts of these heterologous P.II are also precipitated (for example, P.IIb, P.IIc, P.IId, and P.IIe with αJS3 a* serum). The antiserum raised against strain JS5 f* Gc exhibits a prominent reaction with JS3 P.IIb. Note also the cross-reaction demonstrated between αJS3 e* serum and P.IIb of the same strain. Note the small but discernible immunoprecipitating reactivities of all antisera with P.IIe and P.IIb of strain JS3.
organisms of the same strain (JS3). Fig. 2 shows the other $^{125}$I-labeled Gc proteins (P.I and P.III) occur in these immunoprecipitates, but that their amounts are minor compared with P.II constituents.

Antigenic differences detectable among different P.II by immunoprecipitation with anti-whole P.II$^+$ Gc sera allowed visualization of P.II constitutions of Gc with multiple P.II (Fig. 3). The Gc (X and Y) used as antigens had been serially selected to have several P.II, but the number and identity of their P.II moieties were not discernible simply by autoradiography after SDS-PAGE of the $^{125}$I-labeled whole Gc lysates. The immunoprecipitation results show the following phenotypes for these two Gc preparations: X, P.IIacde$^+$, Y, P.IIabcd$^+$.

Coagglutination Reactions. Coagglutination was used early in this study as another assessment of reactions between surface-exposed Gc antigens and immune sera. The limited coagglutination data obtained are shown in Table II. In all instances, the most pronounced reaction of a given antiserum was with organisms bearing the same P.II contained in the immunizing preparation. Though this coagglutination technique appears to discriminate among different P.II moieties, its sensitivity suffers by comparison with either immunoprecipitation or immunoblotting, and it was not used for more extensive analysis of antigenicities of P.II.

Immunoblotting with Antisera Raised against Whole P.II$^+$ Gc. The anti-whole P.II$^+$ Gc sera used in the above-described immunoprecipitations were also studied by immunoblotting as shown in Fig. 4. Nearly identical specificities for these antisera with different P.II moieties were found by immunoblotting as compared with immunoprecipitation. Each antiserum (except aJS3 P.IId$^+$) exhibited pronounced reactivity with the homologous P.II; clearest examples of antigenic cross-reactivities are found between JS3 IId and JS5 IIf (with both aJS3 P.IIb$^+$ and aJS5 P.IIf$^+$ sera). The immunoblotting reactions shown in Fig. 4 were obtained after absorption of the antisera with P.II$^-$ organisms (strain JS3), and the influence of absorption on the reactivities of these sera is illustrated in Fig. 5 where preimmune, unabsorbed immune,
and absorbed immune sera from a single animal were compared. No reactions are visible for preimmune serum. Unabsorbed immune serum reacts with many Gc constituents on the nitrocellulose sheet; most of these are not constituents that are readily $^{125}I$-labeled on whole Gc (not shown). The serum shown was raised by immunization with JS5 P.IIf$^+$ organisms, and it reacts with both P.IIf (JS5) and P.IIb (JS3) whether absorbed (with P.II$^-$ Gc) or unabsorbed. Absorption clearly diminished or abolished reactivities of this serum with many non-P.II constituents.

**Immunoprecipitation and Immunoblotting with Sera of Rabbits Immunized with Isolated P.II Material.** Two sera from animals immunized with “purified” P.II constituents were used for immunoprecipitation and immunoblotting as shown in Fig. 4 (bottom two panels) and in Fig. 6. Both of these sera react with all P.II constituents in immunoblotting. One (aP.II$\alpha$, JS1) exhibits rather weak reactivity with one particular P.II (P.II, R10); the other serum (aP.II, R10) reacts well with every P.II used for immunoblotting (Fig. 4). These two anti-P.II sera exhibited much narrower reactivities by immunoprecipitation as depicted for the aP.II$\alpha$ (JS1) serum in Fig. 6. The aP.II$\beta$
Fig. 5. Comparisons of preimmune with nonabsorbed and absorbed immune serum by immunoblotting. Serum was collected from a single rabbit before its immunization (Pre); serum was collected after an immunization course with whole JS5 P.II* organisms and used before (Imm) and after (Abs-Imm) absorption with JS3 P.II- Gc. The whole P.II* Gc used as antigens are noted on the top (their autoradiographic profiles are shown in top panel of Fig. 3). Also included in each blot is a transfer control (TC) consisting of 12sI-labeled JS5 P.II- Gc that is also useful for reference by virtue of the radioemission of its P.I and P.III bands. With preimmune serum, IMle or no reactivity is noted in the immunoblot. With unabsorbed immune serum (Imm), multiple bands appear to bind antibody; the most intensely reactive constituents are the homologous P.IIf and P.Iib of strain JS3. These same P.II are recognized by absorbed immune serum (Abs-Imm), which now fails to bind to other, non-P.II constituents in the blot of whole Gc lysates. All sera used at 1:100 dilutions.

(JSI) serum immunoprecipitates best the homologous P.IIf; several other P.II (P.IIa of JS1, P.IIg of JS5, P.IIi of R10, and P.IIb, P.IIc, and P.IIe of JS3) show weak, definite immunoprecipitation by this serum. The other anti-P.II serum (αP.II), R10 reacts with all P.II moieties by immunoblotting (Fig. 4); its immunoprecipitation reactivity (not shown) was mainly with the homologous P.IIf but included a minor reaction with P.IIa (JS1). The broad reactivities of these sera in immunoblotting permit visualization of P.II constituents of Gc in individual colonies whether single or multiple P.II were present. This is shown in Fig. 7 where single JS3 colonies of diverse P.II* phenotypes were used as antigens in immunoblotting with the αP.II serum. The results suggest that the quantity of a given P.II (P.IIa for example) is about the same when it is the only P.II present as when it occurs concomitant with one, two, or several other P.II.

Heat-modifiability and Antigenic Reactivities of P.II. P.II of Gc display different migra-
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Fig. 6. Comparison of immunoprecipitation and immunoblotting reactions with anti-P.IIβ (JS1) serum. Antiserum from a rabbit immunized with P.IIβ bands excised from SDS-PAGE gels was used for immunoprecipitation (IP) and immunoblotting (IB) against Gc of four different strains. The top panel (WC) is the autoradiograph after SDS-PAGE and transfer to nitrocellulose of 125I-labeled Gc of the same phenotypes as those used for immunoblotting and immunoprecipitation as noted at top. In immunoprecipitation, two preparations (Iαβαβ and Iββα) of the homologous strain were assayed; only the homologous P.IIβ band is prominently immunoprecipitated although minor cross-reactions can be seen (with P.IIα of JS1, P.IIγ of JS5, P.IIδ of R10, and P.IIβ, P.IIγ, and P.IIε of JS3). By immunoblotting, all P.II show considerable reactivity with this antiserum except P.IIγ of R10 where the reaction is quite weak. Note that only the Iαβαα derivative of strain JS1 was used for immunoblotting; also included in the blot is a transfer control (TC) of 125I-labeled JS5 P.II− organisms in which P.I and P.III are visible.

Comparison of Immunoprecipitation and Immunoblotting Results. Summaries of the reactivities for anti-whole P.II+ Gc sera and anti-P.II+ sera assessed by immunoprecipi-
Immunoblotting of individual P.II+ Gc colonies with anti-P.II serum. Individual colonies of strain JS3 with desired P.II phenotypes were lifted from solid growth medium, immersed in SDS-solubilizing solution, boiled, and each loaded into separate wells for SDS-PAGE. After electrophoresis and transfer to nitrocellulose, the blots were incubated with anti-P.II JS1 serum (1:500). Six colonies (1–6) with multiple P.II were obtained by starting with a P.IIa+ colony and serially selecting variants that were more opaque than their parental colonies. These organisms are seen to have the following P.II phenotypes: 1, P.IIabc+; 2, P.IIabcde+; 3 and 4, P.IIad+; 5 and 6, P.IIabcde+. Note that each P.II is apparently present in approximately the same amount in those specimens bearing multiple P.II as in specimens having a single P.II.

Fig. 8. Immunoblotting of P.II after solubilization at different temperatures. Both 125I-labeled Gc (WC, top panel) and unlabeled Gc bearing different P.II phenotypes (P.IIa+ ... P.IIe+) were solubilized in preparation for SDS-PAGE at either 40°C for 30 min or by boiling (100°C) for 10 min. Both the radiolabeled and unlabeled specimens were transferred to nitrocellulose after SDS-PAGE. The nitrocellulose sheet with 125I-labeled lysates was dried and used for autoradiography. The nitrocellulose sheet with unlabeled lysates (except the transfer control, TC) were incubated with aP.II JS1 serum (1:100) and subsequently with 125I-protein A. Two apparent subunit sizes of each P.II are seen (Φ). For most of these P.II (P.IIb, P.IIc, P.IId, P.IIe), only the heat-modified (higher apparent size) form binds appreciable antibody plus 125I-protein A; the exception is protein IIa, both of whose forms give positive immunoblot reactions. The unmodified (lower apparent size) forms of P.IIc and P.IIe exhibit some reaction with the serum, but their intensities are clearly not proportional to the apparent amount of each transferred to nitrocellulose (judging from the autoradiograph of 125I-labeled organisms).

Immunoprecipitation and immunoblotting are presented in Tables III and IV. Immunoprecipitation and immunoblotting results were generally in agreement for sera raised against whole Gc; major differences in reactivity by immunoprecipitation and immunoblotting were found with antisera from rabbits immunized with isolated P.II as described above.

Discussion

The immunological reactivities of P.II demonstrated in this study by immunoprecipitation and immunoblotting have several implications. First, each P.II shares...
TABLE III
Summary of Immunoprecipitation Results

| Antisera   | JS1 | JS5 | R10 | JS3 |
|------------|-----|-----|-----|-----|
|            | Iα  | Iβ  | IIα | IIf | Iig | IIg | IIj | IJf | IIf | IIg | Ili | Ij | Iλ | Ile |
| aJS1 Iαa  | (++)* | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS5 IIa  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS5 IIG  | ±  | ±  | (++) | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS3 IIA  | N  | N  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS3 IIb  | N  | N  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS3 IIc  | N  | N  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS3 IID  | N  | N  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS3 IIE  | N  | N  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aIIA, JS1 | ±  | (++) | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aIIJ, R10 | +  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |

* Parentheses indicate homologous reactions.
† Not done.

TABLE IV
Summary of Immunoblotting Results

| Antisera   | JS1 | JS5 | R10 | JS3 |
|------------|-----|-----|-----|-----|
|            | Iα  | Iβ  | IIα | IIf | Iig | IIg | IIj | IJf | IIf | IIg | Ili | Ij | Iλ | Ile |
| aJS1 Iαa  | (+)* | ++ | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS5 IIa  | ±  | ±  | (++) | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  | ±  |
| aJS5 IIG  | ±  | ±  | (++) | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |
| aJS3 IIA  | −  | ±  | ±  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |
| aJS3 IIb  | −  | ±  | ±  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |
| aJS3 IIc  | −  | ±  | ±  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |
| aJS3 IID  | ±  | ±  | ±  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |
| aJS3 IIE  | ±  | ±  | ±  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  | −  |
| aIIA, JS1 | ++ | (++) | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| aIIJ, R10 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ |

* Parentheses indicate homologous reactions.

Antigenic determiners with most other P.II; these common P.II antigens are seen by immunoblotting with αP.II antisera raised against denatured, purified P.II material. This finding strongly suggests that portions of all P.II have similar or identical portions in their primary structures as suggested previously by 125I-labeled peptide mapping (5). Although these antisera raised against isolated P.II contain antibody molecules directed toward several antigenic portions of nearly all P.II (as shown by immunoblotting), reactions with P.II in situ are restricted to the homologous P.II. Antigenic dissimilarities among different P.II are shown with antisera raised against whole P.II* Gc by both immunoprecipitation and immunoblotting. These whole cell antisera contain anti-P.II antibodies that are apparently directed mainly against exposed portions of the homologous P.II. The exposed portion(s) of each P.II is not totally unique since cross-reactions are found among a few different P.II by immunoprecipitation; but, in general, a given P.II seems to expose portions of its structure.
that is more different than similar as compared with other P.II. Thus, although all Gc outer membrane P.II share common antigenic determiners, these are not the antigens that are exposed on the intact Gc surface. Rather, each different P.II displays a different antigenic facade on the organism's exterior.

The above interpreted findings suggest that P.II are potential contributors to serologic reactivities of outer membrane vesicles or fragments as well as of whole Gc. It seems likely that a serotyping scheme based on P.II antigenicities could be established and would probably include something like 5–10 serologically definable groups if nondenatured P.II were used as antigens. It is not presently clear whether or not the "class J" coagglutination of Danielsson and Sandström (19) is based on P.II constitution. Some of the class J reactions are pronase sensitive, and this suggests that a pronase-sensitive protein is involved in the serologic reaction; however, all P.II are susceptible to trypsin or α-chymotrypsin cleavage on intact Gc and some P.I moieties are also susceptible in situ (21), so these data do not clearly implicate P.II as the class J serotyping antigens.

It is not clear why the heat-modified forms of P.II appear to be better antigens than unmodified forms in immunoblotting. This difference may reflect variation in efficiencies of electrophoretic transfers to nitrocellulose or heat plus SDS-induced changes in availability of antigenic reactive groups to antibodies or both. However, it is clear that such estimations of P.II antigenicity by immunoblotting should be done with preparations that are boiled in SDS before gel electrophoresis.

As mentioned in the introduction, the comparative antigenicities of P.II are of interest because they are surface-exposed constituents of the Gc outer membrane and because Gc change their P.II constitutions with great frequency in vitro; the extant observations on clinical isolates suggest that similar changes in P.II phenotype and colony opacity also occur in vivo (12, 22). There are several implications of these findings. First, Gc populations may present ever-changing antigenic facades to the host's immune system. This might complicate the host's mounting curative and/or protective antibody or cellular immune responses to infecting Gc. Second, because different P.II expose different antigenic moieties on the Gc exterior, it seems likely to expect that these P.II exert differing influences on the surface-mediated or influenced reactions of Gc with host components. A recent study suggests that different P.II constituents confer varied adherence properties on Gc (3). These results plus the finding of several colony opacity/P.II phenotype variants in individual female patients (22) strongly suggest that every P.II phenotype (P.II−, P.IIx+, P.IIxy+, etc.) might have a slightly different role in initiation, maintenance, and progression of local Gc infections.

Summary

Immunoprecipitation or coagglutination with whole gonococci and immunoblotting have been used to assess the comparative antigenicities of 11 different protein II (P.II) moieties from four different strains. Rabbit antisera used for these studies include both anti-whole gonococcal sera and antisera raised by immunization with "isolated" P.II preparations. The results show that: (a) immunization with gonococci possessing a single P.II elicits formation of antibodies directed mainly at the homologous P.II when assessed by either immunoprecipitation or immunoblotting; and (b) immunization with isolated P.II material elicits formation of antibodies that are cross-
reactive with all (or nearly all) P.II species in immunoblots; these antibodies recognize mainly the homologous P.II by immunoprecipitation. These results have been interpreted as showing the following: (a) all gonococcal outer membrane P.II moieties share antigenic determiners, but these common antigens are not generally accessible on the organisms' surfaces for interaction with antibody molecules; and (b) the surface-exposed antigens of different P.II constituents are, in general, different from one another.

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