INFECTION AND IMMUNITY, Dec. 1981, p. 804-816
0019-9567/81/120804-13$02.00/0

Vol. 34, No. 3

Surface-Exposed Protein Antigens of the Gonococcal Outer Membrane

JOHN SWANSON

Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, Hamilton, Montana 59840

Received 26 May 1981/Accepted 17 August 1981

Whole, ^{125}I-labeled gonococci (GC) were incubated with rabbit sera against whole GC. After washing, the [^{125}I]GC were lysed in detergent, and radioiodinated antigen-antibody complexes were immunoprecipitated by protein A-Sepharose. Several GC outer membrane proteins, including proteins I, II, and III, could be identified in immunoprecipitates obtained with these antisera. In many immunoprecipitates, a 44K protein was present. Reactivity of antisera toward protein II could be demonstrated, and some rabbit sera contained very prominent apparent antibody activity toward this protein. Proteins I and III were found in similar ratios in immunoprecipitates, suggesting that they form heteropolymers in GC outer membranes. Qualitative and quantitative differences in antibody reactivity and specificity could be demonstrated with serially obtained sera from rabbits immunized with whole GC. The use of viable or Formalin-fixed heat-killed staphylococci yielded "non-immunological" precipitation of ^{125}I-labeled GC constituents; this occurred with staphylococci regardless of whether they contained protein A.

Many interactions of gonococci (GC) with their external milieu are undoubtedly affected or mediated by surface constituents of the organisms. Surface-surface interactions between GC and neighboring procaryotic or eucaryotic cells are likely determined by surface-exposed moieties of the interacting cells. Pili have been studied as mediators of attachment of these bacteria to several kinds of epithelium-derived cells, erythrocytes, etc., and these proteinaceous cell wall appendages are currently viewed as promoting virulence of GC by enhancing their attachment and colonization on mucosal surfaces (2, 6, 27). Outer membrane proteins are also exposed on GC surfaces and have been implicated as promoters of both intergonococcal adherence and attachment of GC to both tissue culture cells and peripheral blood neutrophils in vitro (9, 27, 30). Two "sets" or families of proteins predominate in the outer membrane, and these are currently called proteins I and II (29). Previous studies have utilized exogenously added proteolytic enzymes to probe the properties of these GC outer membrane proteins (1, 28). In the present study, immunoprecipitation techniques have been used to gain information on the exposure and antigenicity of surface-exposed, outer membrane proteins of GC. [^{125}I]GC were incubated with immune rabbit sera, and the organisms were subsequently lysed and subjected to immunoprecipitation and electrophoresis in polyacrylamide gels to visualize those constituents of the whole cells to which antibodies had attached.

MATERIALS AND METHODS

GC. GC strains JS1, JS2, and JS3 were grown on clear typing medium previously described (10) but modified here by the substitution of Difco agar (Difco Laboratories, Detroit, Mich.) for Noble agar (BBL Microbiology Systems, Cockeysville, Md.). Organisms were grown at 36°C in a 5% CO_2 incubator for 21 to 23 h before use. Only nonpiliated (P^-) organisms were used in this study. Opaque (O^{++}), intermediate opaque (O^+), and transparent (O^-) colony forms were serially passaged by selection of single colonies of desired phenotype as described previously (23).

Radioiodination of GC. GC were swabbed from solid medium and suspended in Dulbecco phosphate-buffered saline (DPBS [5]), pH 7.3, to a turbidity of 200 Klett units (blue filter). A 1.5-ml sample of this suspension was centrifuged, and the pellet was resuspended in 40 μl of DPBS to which was added 5 μl of 10^{-5} M KI. This thick suspension was transferred to a glass screw-top vial previously coated with 10 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodo-Gen; Pierce Chemical Co., Rockford, Ill.) as 10 μl of a 1-mg/ml solution in chloroform which was evaporated under a nitrogen stream (15). After the addition of the GC suspension containing KI, 500 μCi of ^{125}I (as ^{125}INa,

in water, 100 μCi/μl) was added, and the mixture was incubated at room temperature with occasional shaking for 10 min. The contents of the vial were quantitatively transferred to a 1.5-ml Microfuge tube containing 1 ml of cold DPBS, and after centrifugation (Microfuge B, top speed, approximately 3,650 × *g*, 1.5 min) the pellet was washed twice each with 1 ml of cold DPBS.

Immunoprecipitation. The final washed pellet of [^{125}I]GC was suspended in 450 μl of DPBS, and this was dispensed as 80-μl portions into 1.5-ml Microfuge tubes, to each of which was added 40 μl of serum. The serum-[^{125}I]GC mixture was incubated in an ice bath for 20 min with occasional mixing, after which the pellet was collected by centrifugation (Microfuge, 1.5 min) and washed twice each with 1 ml of ice-cold DPBS. The washed pellet was suspended in 205 μl of either 1% Triton X-100 (TX100) in DPBS or 1% *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14 [Zw314]; Calbiochem-Behring Corp., La Jolla, Calif.) in DPBS. This mixture was thoroughly mixed, incubated at 40°C for 1 h, and centrifuged (Microfuge B, 4 min, top speed). A 190-μl portion of the supernatant was carefully removed and transferred to a 1.5-ml Microfuge tube containing protein A-Sepharose 4B CL (Sigma Chemical Co., St. Louis, Mo.) (40 μl of a slurry containing the equivalent of 2 mg of the dry protein A-Sepharose conjugate previously swollen overnight at 4°C in DPBS). This mixture was incubated for 15 min at room temperature with end-over-end rotation. After a brief (30-s) centrifugation (Microfuge), the loose pellet was washed twice each with either 1 ml of 1% Zw314 in DPBS or 1 ml of 1% TX100 in DPBS depending on which detergent had been used to lyse the organisms. At each wash a small amount (approximately 10 μl) of wash solution was left overlying the pellet. After the last wash, all excess fluid was removed from the pellet by aspiration, and the pellet was either suspended in 1 ml of absolute ethanol or directly solubilized by boiling in 20 μl of 4% sodium dodecyl sulfate (SDS) (specially pure sodium lauryl sulfate; British Drug House; obtained through Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.), 8% 2-mercaptoethanol, 20% glycerol, 0.125 M tris(hydroxymethyl)aminomethane (pH 6.8), and bromphenol blue. The alcohol solution was left at −30°C for 1 h and centrifuged, the supernatant was aspirated, and the pellet was dried in a boiling water bath before solubilization in 20 μl of the same SDS-containing solubilizing solution noted above. The entire slurry was loaded into a slab gel slot of a 12.5% acrylamide gel formulated according to the recipe of Laemmli (13). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 40 mA constant current, and the slab was fixed in 25% 2-propanol–7% acetic acid for at least 2 h before use for autoradiography. Autoradiograms were obtained by exposing X-ray film (Kodak XAR2) to the wet, fixed gel enclosed in a plastic bag. Some gels were first dried and then applied to X-ray film (Kodak XAR2 or XTL) and exposed with an intensifying screen at −70°C in a metal casette or at room temperature in a cardboard X-ray folder. Autoradiograms were analyzed both by visual inspection and by scanning on a Joyce-Loebl microdensitometer equipped with an integrating attachment.

Use of staphylococci in immunoprecipitation. *Staphylococcus aureus* Cowan I, Wood 46, RN 450, and RN 25 strains were obtained from Alan Barbour (Rocky Mountain Laboratories), as was a strain of *Staphylococcus epidermidis*. These were prepared according to the recipe of Kessler (12) and were used as 10% suspensions of Formalin-fixed, heat-killed organisms. In these studies, 10 to 20 μl of staphylococcal suspension was used for the precipitation of [^{125}I]GC proteins either with or without prior exposure of the [^{125}I]GC to rabbit antisera, as described above for protein A-Sepharose 4B CL. In a few experiments, whole viable staphylococci were utilized. These were prepared by obtaining a pellet of staphylococci grown either on the clear, solid GC medium noted above or in beef heart infusion broth overnight and then suspending the pellet to yield a 10% suspension (wet wt/vol) which was used for immunoprecipitation.

Rabbit antisera. GC of desired strain and colony opacity were scraped from solid medium plates and suspended to a turbidity of 200 Klett units in sterile DPBS. These organisms were washed twice with cold DPBS by centrifugation. Final, washed pellets were suspended to the original turbidity, and this suspension was divided and frozen. Portions (0.5 ml) were thawed and inoculated into rabbits twice subcutaneously at 2-day intervals and then intravenously every 2 to 4 days for a month (nine total intravenous inoculations). At 5 weeks after this month's immunization schedule, the rabbits were immunized again intravenously with 0.5 ml of the suspension noted above. Three rabbits were used for immunization with each strain and each of two opacity phenotypes (O^{++} and O^{-}). Sera were collected and kept at −30°C until used.

RESULTS

Nomenclature and properties of gonococcal outer membrane proteins. Different investigators or research groups have studied the outer membrane proteins of GC and have used several systems of nomenclature for the proteins as they are displayed by SDS-PAGE. Some of these terms are included in Table 1, which also includes the protein designations used here.

Use of the names proteins I and II follows the recommendations from a recent workshop on pathogenic *Neisseria* (29). Protein I (designated P.I in the figures) was present in all GC examined to date, it had the same apparent subunit molecular weight (MW) for intrastrain derivatives (Fig. 1), its subunit MW varied from strain to strain (Fig. 2), and it exhibited no heat or 2-mercaptoethanol modification in apparent size by SDS-PAGE (Fig. 1). Protein II (P.II) species were prominent on O^{++} and O^{+} GC (Fig. 1) and displayed increases in their apparent MW upon boiling before SDS-PAGE (Fig. 1 and 2) (26). This heat modifiability (Fig. 1) occurred with or without 2-mercaptoethanol and resembled prop-

TABLE 1. *Protein designations by various authors*

Designations used here for outer membrane proteins	Outer membrane protein designations by the following authors:				
	Swanson (24-26)	Buchanan and Pearce (2)	McDade and Johnston (16)	Heckels et al. (7,8,14)	Newhall et al. (18)
Protein I (P.I)	Major protein (MP)	Principal outer membrane protein (POMP)	Protein 1	Protein I	Principal outer membrane protein (POMP)
	Major outer membrane protein (MOMP)				
Protein II (P.II)	Minor protein (mP)		Protein 2,2*	Protein II,II*	Opacity-associated protein (OAP)
	Heat-modifiable proteins (hmP) (hmP_{OP}, hmP_{LA})				
Protein III (P.III)			Protein 3,3*		

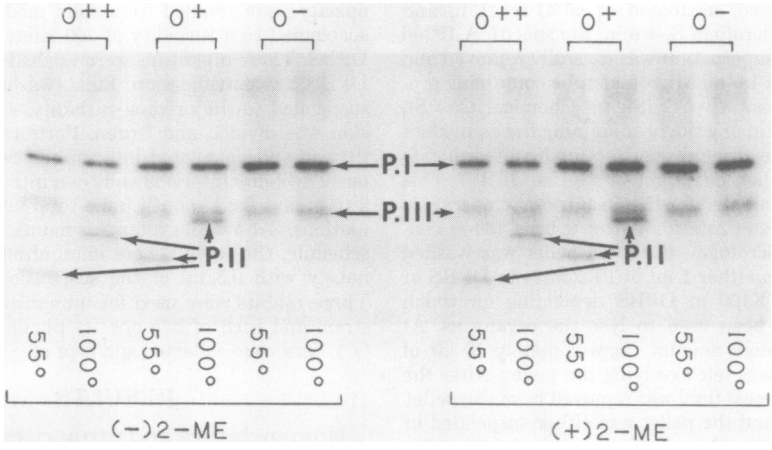

FIG. 1. *Influences of heating and 2-mercaptoethanol on migration characteristics of ^{125}I-labeled GC proteins I, II, and III in SDS-PAGE. GC of strain JS1 with opaque (O^{++}), intermediate opaque (O^{+}), and transparent (O^{-}) colony characteristics were ^{125}I labeled and suspended in 4% SDS. Each specimen was divided and solubilized at 55°C or at 100°C in the absence [(−)2-ME] or the presence [(+)2-ME] of 8% 2-mercaptoethanol as shown. Note the equivalent intensities and migration distances for protein I species in all specimens. Protein III exhibited a slight decrease in apparent subunit MW in the presence or absence of 2-ME and was unchanged at boiling versus lower temperature for solubilization. Protein II exhibited a lower apparent subunit MW in specimens solubilized at 55°C as compared with those solubilized by boiling. Note that this figure and Fig. 2, 5a, 6a, 8, and 9a represent only the central portions of autoradiograms obtained after SDS-PAGE.*

erties described for the *ompA* protein of *Escherichia coli* (3, 7). No provision was made in the above-noted recommendations for the protein herein called protein III (P.III), which is probably the same as protein 3,3* described by McDade and Johnston (16). In their studies, this protein exhibited iodination on intact GC radiolabeled with lactoperoxidase and exhibited a slight increase in apparent MW on reduction with 2-mercaptoethanol. In my laboratory, observations paralleling those of McDade and Johnston on the modifiable MW of this protein have been obtained by using isolated outer membranes (R. Judd and J. Swanson, unpublished observations). When whole [^{125}I]GC were subjected to SDS-PAGE in the presence and absence of 2-mercaptoethanol, little change in apparent MW was found for this protein (Fig. 1).

Light microscopic observations. GC that had been ^{125}I labeled, washed, incubated with rabbit serum, and washed were compared with untreated GC by phase-contrast microscopy at each step. No striking "ballooning" or other distortions in morphology were seen in treated

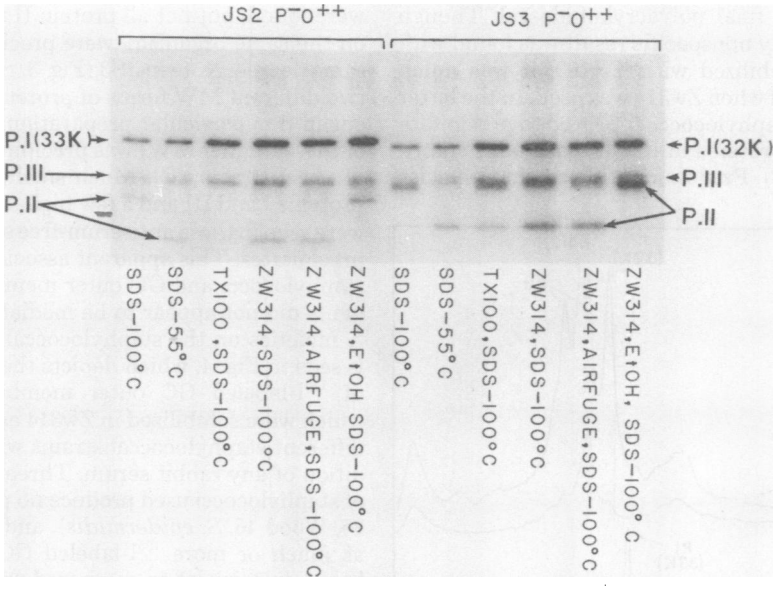

FIG. 2. *Solubilization of [^{125}I]GC proteins by SDS, TX100, and Zw314. GC with colony phenotypes P⁻O⁺⁺ from strains JS2 and JS3 were ^{125}I labeled and incubated with detergents as shown. After centrifugation the supernatants were analyzed by SDS-PAGE after solubilization either at 55°C or by boiling. The TX100 and Zw214 extracts were all boiled in SDS before SDS-PAGE. The Zw314 extract of each strain was subjected to centrifugation in a Microfuge B or in an Airfuge, and the supernatants were prepared for SDS-PAGE as described in the text. Note the different apparent subunit MWs for protein I of JS2 (33K) and for protein I of JS3 (32K). TX100 extracts appeared to contain less protein II than either Zw314 or SDS extracts. Little or no difference in intensity was found for any of the ^{125}I-proteins after ultracentrifugation (Airfuge) as compared with that after lower-speed centrifugation. Although TX100 and Zw314 extracts were boiled with SDS before SDS-PAGE, protein II species exhibited MWs typical for boiled SDS lysates only when these detergent-solubilized specimens were first subjected to ethanol precipitation (EtOH). When this ethanol step was omitted, protein II showed migration characteristic of SDS lysates solubilized at 55°C.*

versus untreated organisms. In contrast, GC that had been incubated for only 15 min at 4°C in phosphate-buffered saline lacking calcium and magnesium exhibited marked enlargement and rarefaction of their internal structure (data not shown). These observations suggest that GC integrity was relatively well maintained during the manipulations of radiolabeling, reaction with antiserum, etc.

Detergent extraction and electrophoretic characteristics of [^{125}I]GC outer membrane proteins. After incubation of [^{125}I]GC in detergent (SDS, TX100, or Zw314), the radioiodinated proteins present in the supernatant of the detergent lysates were compared by SDS-PAGE. TX100 and Zw314 seemed to extract similar amounts of protein I from both strains of GC shown in Fig. 2. TX100 and Zw314 differed in that TX100 extracted, or solubilized, less protein II than did the zwitterionic ionic detergent. ^{125}I-labeled proteins that are qualitatively and quantitatively similar were found in supernatant fractions of Zw314 lysates after Microfuge (approximately 3,650 × *g*, 4 min) versus Airfuge (165,000 × *g*, 30 min) centrifugation. Present in the SDS lysate were two forms of protein II, depending on whether 55°C or boiling temperatures were used to prepare the lysates for SDS-PAGE. At 55°C, protein II exhibited a smaller apparent subunit MW than when the specimens were heated in a boiling water bath. In both TX100 and Zw314 lysates which were boiled in SDS before SDS-PAGE, protein II species migrated at the same lower-apparent-MW form seen for 55°C. However, when the Zw314 lysate (Fig. 2) or a TX100 lysate (data not shown) was treated with absolute ethanol before preparation for SDS-PAGE (boiling in SDS), the higher-apparent-MW form of protein II was again present. The behaviors of proteins I and II of strain JS1 with regard to their extractibilities by TX100 and Zw314 and to their migration characteristics in SDS-PAGE were similar to those described above and shown in Fig. 2 for JS2 and JS3.

Use of staphylococci in immunoprecipitation. Initial experiments were carried out with viable or Formalin-fixed staphylococcal (Cowan I). Use of these organisms yielded relatively high background amounts of ^{125}I-labeled GC compo-

nents in the final polyacrylamide gel. Though this seemingly nonspecific result was found with [^{125}I]GC solubilized with TX100, it was much more marked when Zw314 was used. In the latter detergent, staphylococci appeared to precipitate several GC proteins in the absence of any antiserum (Fig. 3). Predominantly protein II species were found, but not all protein II subunits, even on the same organism, were precipitated to the same degree. Note in JS3 (Fig. 3, tracing C') that two different MW forms of protein II were present in this particular preparation, but only one of these (higher MW) was precipitated by staphylococci (tracing C). Much smaller amounts of proteins I and III and a few higher-MW proteins were seen in these antiserum-free staphylococcal precipitates. This apparent association between staphylococci and GC outer membrane components did not appear to be mediated by protein A moieties on the staphylococcal surface. This is seen in Fig. 4, which depicts the precipitation of ^{125}I-labeled GC outer membrane proteins which were solubilized in Zw314 and mixed with different staphylococcal strains without the addition of any rabbit serum. Three of the strains of staphylococci used produce no protein A (RN 25, Wood 46, *S. epidermidis*), and these yielded as much or more ^{125}I-labeled GC outer membrane proteins when compared with two staphylococcal strains (RN 450 and Cowan I) that produce protein A.

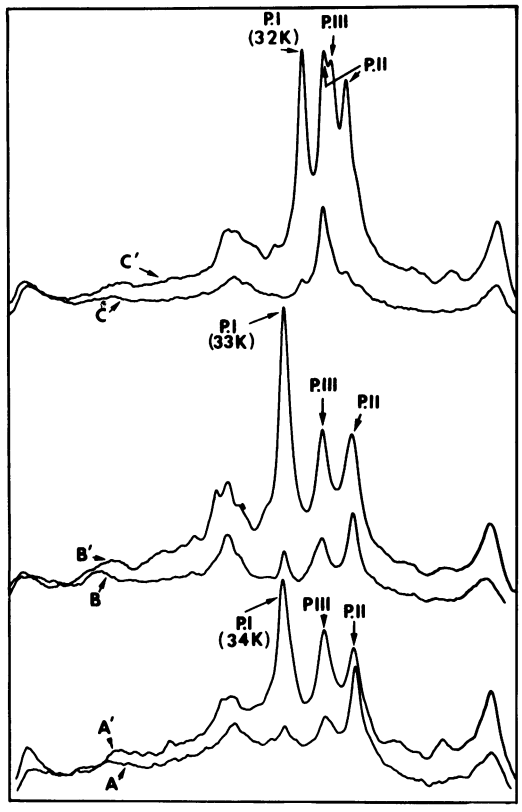

FIG. 3. *Antiserum-independent precipitation of ^{125}I-labeled GC proteins by Cowan I staphylococci. P^-O^{++} gonococci from strains JS1, JS2, and JS3 were radioiodinated, lysed in Zw314, and reacted with S. aureus Cowan I strain without the addition of antiserum. The densitometric tracings of autoradiograms after SDS-PAGE of the Zwittergent lysate supernatants are shown in A' (JS1), B' (JS2), and C' (JS3). Densitometric tracings of autoradiograms after SDS-PAGE of the staphylococcal pellets from lysates of the three gonococcal strains are also shown (A [JS1], B [JS2], C [JS3]). For all strains, small amounts of proteins I and III were present after precipitation with staphylococci, but the predominant radioemitting bands in each precipitate were protein II moieties. Note that with JS3 P^-O^{++} organisms in which two protein II species with different subunit MWs are present in the whole Zw314 lysate (C'), only one protein II band (*) is found in the staphylococcal precipitate (C). (The densitometric tracings in this and all following figures are oriented with the top of the gel to the viewer's left.)*

Immunoprecipitation results with protein A-Sepharose. Substitution of protein A-Sepharose for whole staphylococci yielded much

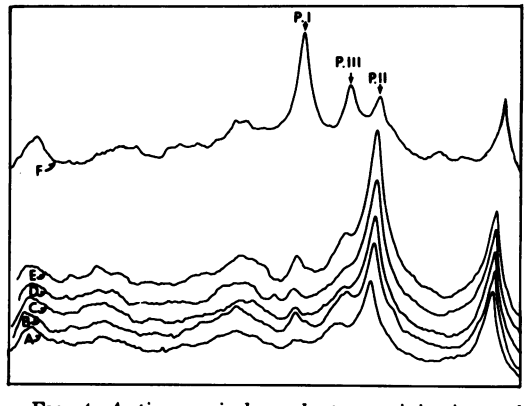

FIG. 4. *Antiserum-independent precipitation of ^{125}I-labeled GC proteins by different strains of staphylococci. P^-O^{++} GC from strain JS1 were ^{125}I labeled, lysed in Zw314, and then mixed with Formalin-fixed, heat-killed staphylococci without prior incubation with serum. All of the staphylococcal pellets contained several radioemitting bands derived from GC. Especially prominent were protein II moieties, regardless of which staphylococcal preparation was used. Of the five strains of staphylococci used, slightly greater amounts of protein II were associated with staphylococci lacking protein A (C, S. epidermidis; D, Wood 46; E, RN25) as compared with those staphylococci that produce protein A (A, Cowan I; B, RN450). The densitometric trace of autoradiographic profiles of ^{125}I-proteins present in the Zw314 lysate as resolved by SDS-PAGE is shown (F).*

less nonspecific precipitation of ^{125}I-labeled GC proteins and was used, as described above, for all experiments described below. SDS yielded more complete solubilization of ^{125}I-labeled GC proteins than did TX100 or Zw314, but the immunoprecipitation of radioiodinated constituents was not successful in 0.1% SDS. Therefore, incubation of [^{125}I]GC with rabbit serum was followed by lysis of the organisms in either TX100 or Zw314. Protein A-Sepharose was added to a supernatant of this lysate to precipitate any antibody-^{125}I-antigen complexes present. Use of these techniques for immunoprecipitation of ^{125}I-proteins of GC incubated with rabbit antisera permits several conclusions concerning the immunological reactivities of proteins exposed on GC surfaces, as follows.

(i) Proteins I and II were surface-exposed antigens of GC.

(ii) Similar ratios of protein I to protein III were found in all immunoprecipitates.

(iii) At least one ^{125}I-labeled GC protein (44K) that was present in modest amounts on intact GC was often prominent in immunoprecipitates.

(iv) Semiquantitative changes were demonstrated in the immunoprecipitating activities of sera serially obtained during immunization of individual rabbits.

(v) Qualitative differences in the ^{125}I-proteins immunoprecipitated were found among sera from different rabbits immunized with different strains and intrastrain colony opacity variants of GC.

The above-noted points will be depicted in the following descriptions of a few of the dozens of immunoprecipitation experiments that have been done.

Variation in amount of antiserum used. Dose-response relationships between the amount of antiserum used and the intensity of radioemission by ^{125}I-protein bands in the autoradiogram are shown in Fig. 5. ^{125}I-labeled bands were absent when JS2 GC were incubated with preimmune serum. Increases in intensities of proteins I, II, III and a 44K protein of O^{++} GC were seen with increasing amounts of antiserum raised by immunization with homologous strain, opaque (O^{++}) colony forms. This particular antiserum yielded immunoprecipitates containing much more protein II than protein I. Since protein II is absent or markedly reduced in amount from these transparent colony (O^{-}) organisms, only a change in the intensities of their proteins I and III was seen with variations in amounts of antiserum. The relative intensities of protein II versus protein I bands in the O^{++} GC immunoprecipitates were quite different from those seen in whole cell lysates. This difference was not due to preferential solubilization of protein II, as shown above in Fig. 2. A 44,000-dalton subunit protein was also prominent in the immunoprecipitate autoradiogram and is described below. The relationship between dilution of the antiserum and the amount of particular ^{125}I-proteins in the resultant immunoprecipitates was explored as shown in Fig. 5b. From the shapes of the densitometric curves, it is plain that decisions regarding which represent particular proteins for the purposes of integrating the areas beneath these peaks were somewhat arbitrary. In spite of this, a fairly linear relationship between the peak area ($\log_{10}$) and the antiserum dilution ($\log_2$) was obtained for proteins I and II and the 44K moiety.

Immunoprecipitation of ^{125}I-labeled outer membrane proteins by serially collected sera from individual rabbits after immunization. Immunoprecipitation reactions were carried out with homologous strain (JS1) GC and with sera collected from a single rabbit before and several times after initiation of immunization with JS1 $P^{-}O^{++}$ organisms. Both $P^{-}O^{-}$ and $P^{-}O^{++}$ GC were examined in reactions with these sera from serial bleedings (Fig. 6). Several observations are pertinent. First, no detectable ^{125}I-proteins were immunoprecipitated by the preimmunization serum. After a 1-month (5-week) course of subcutaneous and intravenous inoculations with $P^{-}O^{++}$ organisms, the rabbit serum appeared to react with proteins I, II, and III. A slight decline in amounts of immunoprecipitated ^{125}I-proteins was seen on reaction of radioiodinated GC with sera collected 1 week and 2 weeks after completion of this immunization schedule (6 weeks, 7 weeks). A marked increase in the amounts of ^{125}I-proteins was present in immunoprecipitates generated through use of serum collected 3 weeks after a booster immunization with the same whole (JS1, $P^{-}O^{++}$) GC preparation. The intensities in radioemission of protein I bands from $P^{-}O^{-}$ and $P^{-}O^{++}$ GC obtained after reaction with these serially collected sera were very similar. Because both visual examination and densitometric scans showed roughly similar ratios of proteins I and III regardless of which strain, colony opacity phenotype, or immune serum were used, the seemingly parallel occurrence of proteins I and III was studied in detailed densitometric scans of immunoprecipitate autoradiograms obtained after reaction of JS1 $P^{-}O^{-}$ GC (Fig. 6, C through F) or JS1 $P^{-}O^{++}$ (C′ through F′) with homologous sera obtained from a single rabbit during the course of immunization. The areas representing proteins I and III are quite similar relative to one another in the eight immunoprecipitates (see Fig. 9d). Also, roughly parallel differences in the radioemitting intensities of these

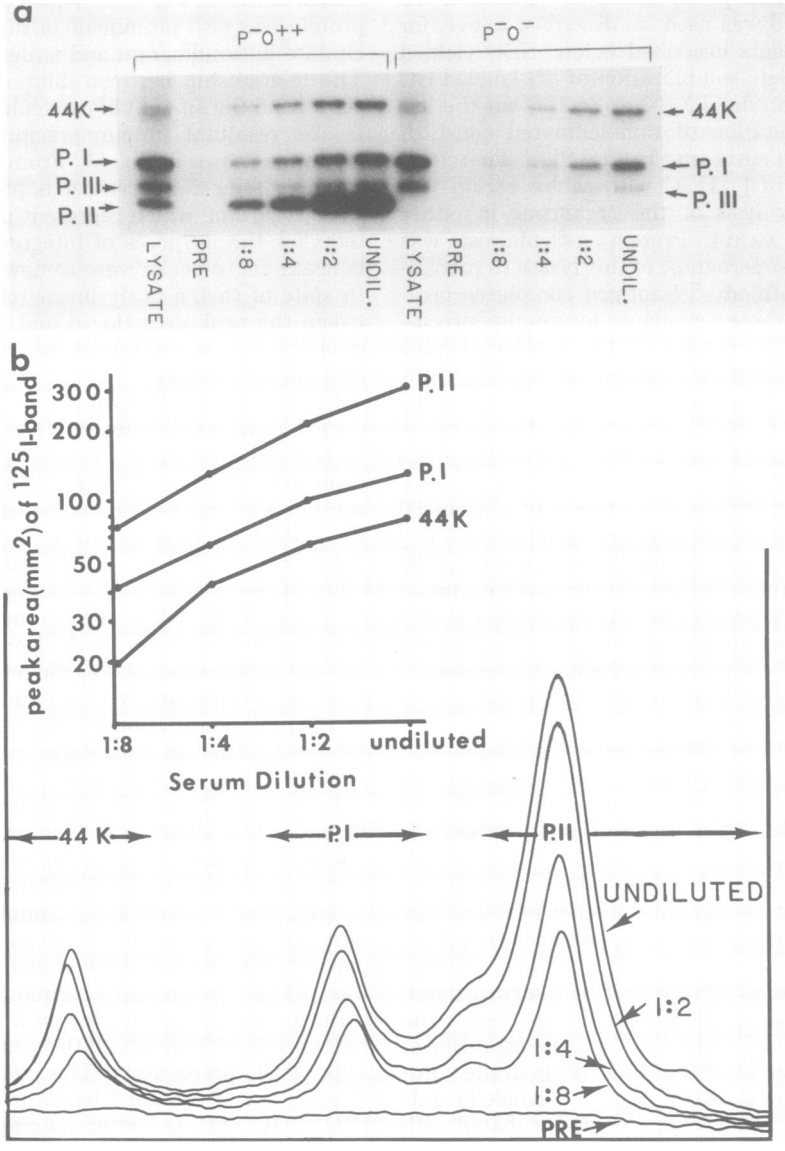

FIG. 5. *Relationships between amount of antiserum and the quantities of immunoprecipitated ^{125}I-labeled GC proteins. Strain JS2 GC with P^-O^{++} and P^-O^- phenotypes were radioiodinated and incubated with rabbit serum obtained before immunization or with a single antiserum preparation raised against homologous strain (JS2) P^-O^{++} GC. This αO^{++} serum was diluted as shown before mixing with [^{125}I]GC. The resultant immunoprecipitates separated by SDS-PAGE are shown after autoradiography in Fig. 5a. Note the heavy ^{125}I-emitting protein II band resulting from incubation of P^-O^{++} organisms with the anti-O^{++} serum; this band is absent in the immunoprecipitates from P^-O^- organisms since these GC lack appreciable amounts of this protein II constituent. As compared with protein I, the amounts of protein II in immunoprecipitates from P^-O^{++} GC reacted with anti-O^{++} serum are greater than those seen in simple SDS lysates of the whole [^{125}I]GC (left lane for both P^-O^{++} and P^-O^-). These immunoprecipitates from P^-O^{++} GC and anti-O^{++} serum were subjected to densitometric scanning, as shown in Fig. 5b. The areas corresponding to protein I, II, and 44K peaks were plotted for immunoprecipitates obtained at each antiserum dilution, as shown in the inset of Fig. 5b.*

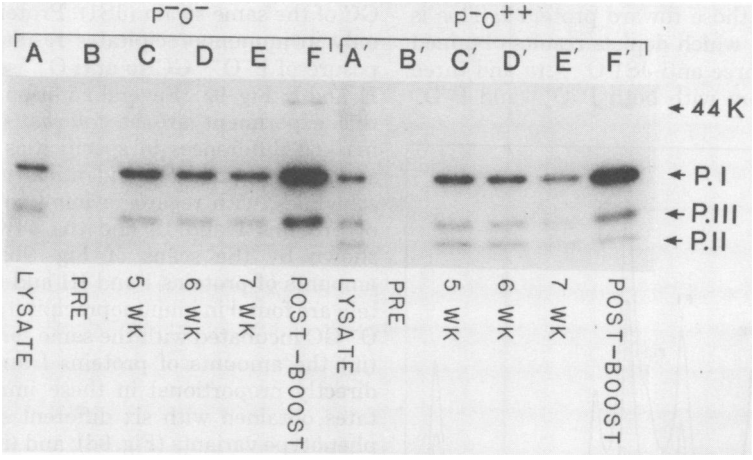

FIG. 6. *Immunoprecipitation with JS1 O^- and O^{++} GC and sera serially collected from a rabbit immunized with JS1 O^{++} GC. A pre-immunization (PRE) serum was obtained from a rabbit which was then subjected to immunization with strain JS1 GC with P^-O^{++} phenotype by the protocol outlined in the text. Sera were obtained at the end of 1 month of immunization (5 wk), 1 week later (6 wk), and 2 weeks later (7 wk) while no immunization was carried out and after booster immunization and a 3-week interval (POST-BOOST). These sera were incubated with homologous strain GC with O^- and O^{++} phenotypes, and the immunoprecipitates are shown after SDS-PAGE and autoradiography. The whole Zw314 lysates of the ^{125}I-labeled O^- and O^{++} GC are also shown. No ^{125}I-labeled bands are found after reaction with preimmune serum. Immunoprecipitates obtained after 5 weeks contained prominent amounts of proteins I and III for both O^- and O^{++} GC and of protein II with O^{++} organisms. The intensities of all these ^{125}I-emitting protein bands diminished over the next 2 weeks (6 wk, 7 wk). After a booster immunization (POST-BOOST), intensities of proteins I and III are very prominent when compared with those of previously obtained sera; however, the protein II band is not so markedly increased by the postbooster serum by comparison with earlier sera. Note the occurrence of the 44K protein in the immunoprecipitates of both O^- and O^{++} GC incubated with the postbooster serum.*

two proteins can be seen in virtually all of the immunoprecipitate patterns of Fig. 5, 7, and 9. The relative amounts of protein I versus protein II in these immunoprecipitates were similar with three of these serially collected sera (lanes C', D', E'), but much less protein II (as compared with protein I) was immunoprecipitated by the postbooster serum (lane F').

Another experiment utilized sera serially collected from a rabbit immunized with whole JS2 P^-O^{++} organisms and reacted for immunoprecipitation with homologous strain JS2 GC of P^-O^{++} and P^-O^- colony phenotypes. The results (Fig. 7) suggest several conclusions. First, the apparent antibody response to protein II in this rabbit was considerably greater than that to protein I. Second, the initial serum obtained after immunization exhibited immunoprecipitation of protein I exceeding that for protein II. This initial apparent ratio of antibodies against protein I versus those against protein II was obviously reversed during the immunization process. Third, this rabbit generated a striking response to a 44K protein which was ^{125}I labeled on whole GC where it was present in small amounts compared with proteins I, II, or III. Immunoprecipitation of this 44K protein was observed in other immune rabbit sera raised to all three strains and O^{++} and O^- colony phenotypes, but was consistently found only in postbooster sera. These observations suggested that qualitatively different responses were generated by rabbits toward different outer membrane proteins present on the same gonococci. This was exemplified with antiserum from a rabbit immunized with JS3 P^-O^{++} and reacted with GC of the same strain and colony opacity (Fig. 8). The intensity of a protein II species completely overshadows that of other proteins in the immunoprecipitate, and the antiserum seemed nearly monospecific by this immunoprecipitation technique. Note also the apparent lack of cross-reactivity of either antiserum (αJS1 or αJS3) with ^{125}I-proteins of the heterologous strain.

Reaction of O^{++} GC with anti-O^- and anti-O^{++} antisera. All the immunoprecipitation experiments described above utilized O^{++} or O^- GC incubated with antisera raised against O^{++} organisms of homologous or heterologous strains. With these antisera, protein II was present in immunoprecipitates from O^{++} organisms and was absent in those from O^- GC; however, that does not demonstrate whether or not antiserum reactivities toward protein II could be

separated from those toward protein I. This is shown in Fig. 9, which depicts results obtained by incubating three anti-JS1 O^- sera and three anti-JS1 O^{++} sera with both P^-O^{++} and P^-O^- GC of the same strain (JS1). Protein II is found only in immunoprecipitates resulting from exposure of P^-O^{++} GC to anti-O^{++} sera (lanes D, E, and F, Fig. 9a). Several additional findings in this experiment are as follows: (i) there are marked differences in specificities of these six sera, all obtained after identical immunization schedules, with regard to immunoprecipitation of proteins I and II, and the 44K protein, as shown by the scans of Fig. 9b; (ii) similar amounts of proteins I and III and the 44K protein are found in immunoprecipitates of O^{++} and O^- GC incubated with the same serum (Fig. 9c); (iii) the amounts of proteins I and III appear directly proportional in these immunoprecipitates obtained with six different sera and two phenotype variants (Fig. 9d); and (iv) there is an inexact, inverse relationship between amounts of protein I and the 44K protein in individual immunoprecipitates (Fig. 9e).

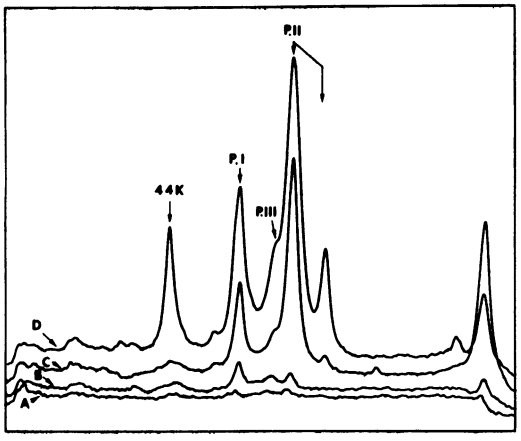

FIG. 7. *Immunoprecipitation with JS2 P^-O^{++} GC and sera serially collected from a rabbit immunized with JS2 P^-O^{++} GC. Homologous GC were incubated with sera obtained before commencing immunization (A), 3 weeks (B) and 5 weeks (C) after starting inoculations, and then after a booster injection and a 3-week rest (D). The densitometric traces of the autoradiograms of these immunoprecipitates are shown. In the first serum sample obtained after starting immunization (B), protein I was more abundant than protein II, but in subsequent sera (C and D) the amount of protein II is much greater than protein I. Note that protein II exhibits incomplete conversion to its higher-apparent-MW form. A prominent 44K band was present in the immunoprecipitate obtained with the postbooster serum (D), whereas little of this band was found in reactions with earlier sera. The peaks of ^{125}I-emitting material at the right of the traces are at the dye front.*

DISCUSSION

The present study was undertaken to design immunoprecipitation assays which will yield information about the antigenicity of surface-exposed proteins residing in outer membranes of GC; two general assumptions have been made regarding the observed results as they relate to these properties of GC outer membrane proteins. First, it is assumed that only proteins exposed on the GC surface are available for interactions with serum antibodies, and that these protein antigen-antibody interactants are then precipitated by protein A-Sepharose. Other GC surface components such as lipopolysaccharide are undoubtedly precipitated in this immunoprecipitation reaction. However, since only proteins are radioiodinated sufficient for their visualization by these techniques, the scope of

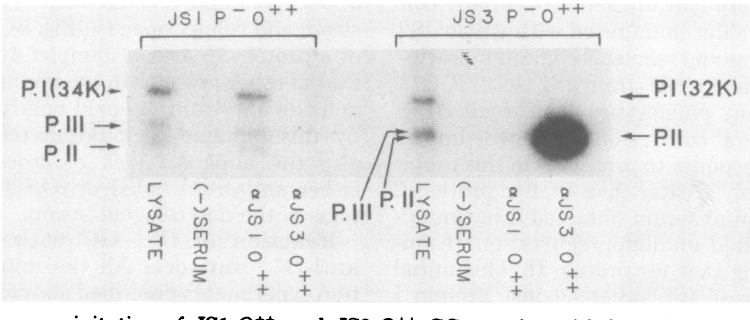

FIG. 8. *Immunoprecipitation of JS1 O^{++} and JS3 O^{++} GC proteins with homologous and heterologous antisera. P^-O^{++} organisms from strains JS1 and JS3 were each incubated with anti-JS1 O^{++} and anti-JS3 O^{++} rabbit sera. The resulting autoradiograms of the immunoprecipitates are shown along with those of whole cell lysates of these organisms, as noted. Proteins I, II, and III were found in the immunoprecipitate of JS1 O^{++} GC incubated with homologous antiserum, but no radioemitting bands were found when the same organism was incubated with antiserum raised to JS3 O^{++} GC. Similarly, ^{125}I-labeled JS3 incubated with anti-JS1 O^{++} serum failed to yield visible radioemitting protein bands after immunoprecipitation. Reaction of JS3 O^{++} GC with this homologous antiserum resulted in apparent immunoprecipitation of only a protein II moiety from these organisms.*

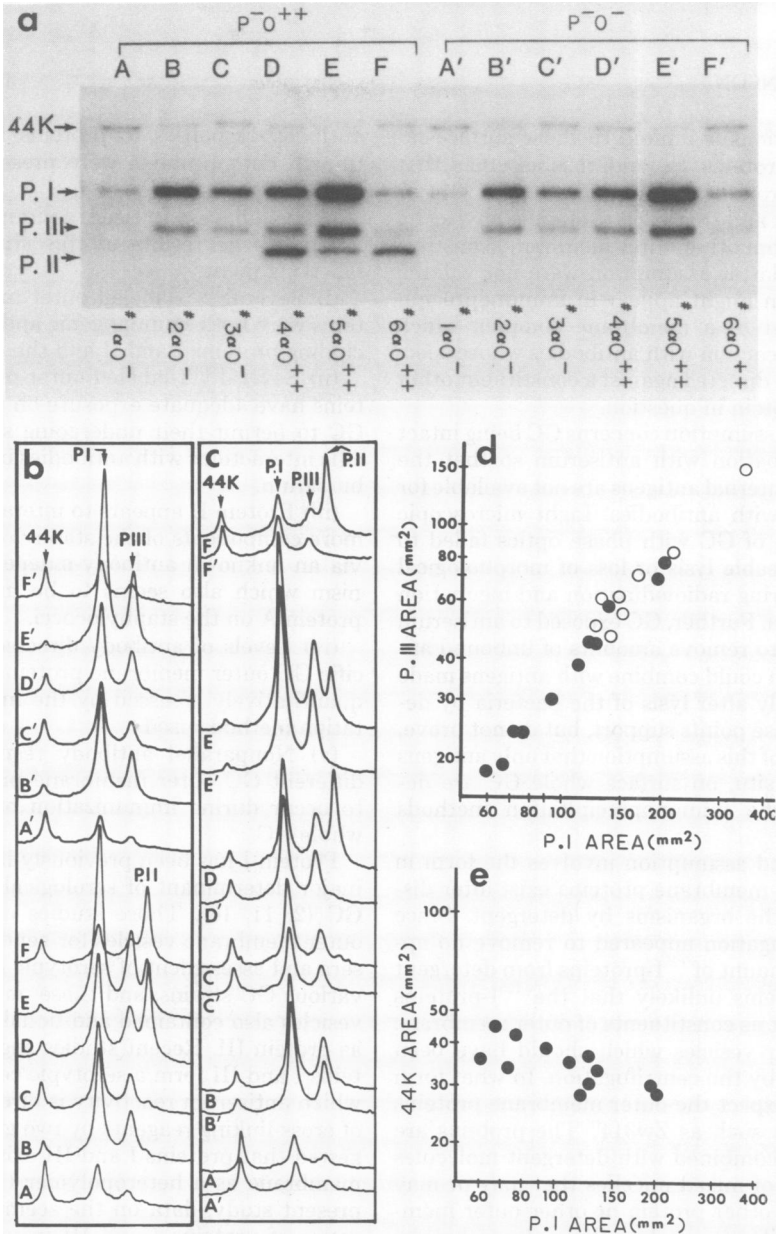

FIG. 9. *Variations in immunoprecipitating activities in sera from different rabbits immunized with JS1 GC. Three rabbits (designated 1 through 3) were immunized with P^-O^- organisms of strain JS1 GC, and three other rabbits (designated 4 through 6) were inoculated with P^-O^{++} organisms from the same strain. After booster immunizations, sera were collected from these six rabbits and were assessed for immunoprecipitating activities against homologous strain GC with O^{++} (lanes A through F) and O^- (lanes A′ through F′) colony phenotypes. The resultant autoradiogram is shown in panel a, and densitometric scans are presented in panels b and c. The arrangement of lanes in panel b is the same as for panel a, whereas the lanes in panel c are arranged so scans of ^{125}I-labeled GC immunoprecipitation products obtained with a given serum and both O^{++} and O^- organisms are paired (A′ and A, etc.). Note the striking variations in intensities of each of the dominant ^{125}I-proteins obtained with the different sera. Antisera from rabbits no. 2 and 5 yielded much greater amounts of protein I in their immunoprecipitates than did antisera from rabbits no. 1 and 6. Protein II was present only in immunoprecipitates of O^{++} GC incubated with anti-O^{++} sera (lanes D through F) and was absent when the same O^{++} organisms were incubated with anti-O^- sera (lanes A through C). Best seen in panel b are the varied amounts of 44K protein and proteins I, II, and III immunoprecipitated by these six antisera. However, the relative amounts of proteins I and III and 44K protein precipitated by any given antiserum were very similar regardless of whether O^{++} or O^- GC were used (panel c, traces A versus A′, etc.). After integration of the proteins I and III and 44K protein peaks of these autoradiograms, the amounts of protein I versus protein III and of protein I versus 44K protein were plotted as shown in panels d and e, respectively. Note the apparent direct, linear relationships between the amounts of proteins I and III in each immunoprecipitate obtained in this experiment (panel d; ●) and in the experiment in Fig. 6 (○). On comparing relative intensities of the protein I and 44K protein bands (panel e), a tendency toward an inverse relationship between these two ^{125}I-proteins is present, but the relationship is not exact.*

my observations is limited to those surface-radiolabeled proteins. Second, it is assumed that individual proteins are solubilized by Zw314 or TX-100 lysis and that each protein is physically separated from other outer membrane constituents. If the latter assumption were not valid, a given protein might appear in immunoprecipitates as part of a membrane complex which underwent reaction with antibodies whose specificities were directed against a constituent other than the protein in question.

The first assumption concerns GC being intact during incubation with antiserum so that the organisms' internal antigens are not available for interaction with antibodies. Light microscopic examination of GC with phase optics failed to reveal noticeable lysis or loss of morphological integrity during radioiodination and incubation in rabbit sera. Further, GC exposed to antiserum are washed to remove amounts of unbound antibody which could combine with antigens made available only after lysis of the bacteria by detergent. These points support, but do not prove, the validity of this assumption that only antigens exposed, in situ, on surface whole GC are detected by the immunoprecipitation methods used here.

The second assumption involves the form in which outer membrane proteins exist after disruption of the organisms by detergent. Since ultracentrifugation appeared to remove no appreciable amount of ^{125}I-proteins from detergent lysate, it seems unlikely that the ^{125}I-proteins were present as constituents of outer membrane fragments or vesicles which should have been sedimented by the centrifugation. In what form would one expect the outer membrane proteins in detergent such as Zw314? The proteins are most likely combined with detergent molecules in the form of mixed micelles that may or may not contain other protein or other outer membrane constituents such as lipopolysaccharide. The results obtained with antisera raised against GC which sport protein II species on their surfaces suggest strongly that protein II does not coexist, primarily, in micelles that also contain protein I constituents. In immunoprecipitation of P^-O^{++} organisms with anti-O^{++} antiserum, protein II was present in the precipitate, whereas use of anti-O^- antiserum raised against organisms lacking protein II failed to immunoprecipitate protein II. The finding that antisera raised against identical O^{++} GC in different rabbits yielded immunoprecipitation patterns in which ratios of protein I and protein II of homologous strain GC are very different also supports the assumption stated above. Further, serially obtained antisera from single rabbits during their immunization revealed apparent, nonparallel antibody responses to proteins I and II even though both proteins were present in the GC used for immunization.

If the above-discussed assumptions can be accepted, the results of this study make the following points.

(i) Several ^{125}I-labeled outer membrane proteins were both immunogenic and antigenic (including proteins I and II and the 44K protein).

(ii) Several ^{125}I-labeled outer membrane proteins have adequate exposure on the exterior of GC to permit their undergoing seemingly specific interactions with antibodies in immune rabbit serum.

(iii) Protein II appears to interact with one or more components of the staphylococcal surface via an unknown antibody-independent mechanism which also seems to be independent of protein A on the staphylococci.

(iv) Levels of antibody directed against specific GC outer membrane proteins can be semiquantitatively assessed by the immunoprecipitation methods used.

(v) Nonparallel antibody responses toward different GC outer membrane proteins appear to occur during immunization of rabbits with whole GC.

Protein I has been previously implicated as a major determinant of serological reactivity for GC (2, 11, 16). Those studies used separated outer membrane vesicles for generation of antisera and assessment of serotypic reactivities for various GC strains, and those outer membrane vesicles also contained additional proteins such as protein III. Recent studies suggest that proteins I and III form a serotypic complex toward which antiserum reactivity is directed (16). Use of cross-linking reagents by two groups has suggested that proteins I and III exist in the outer membrane as a heteropolymer (16, 18). In the present study, data on the seemingly constant ratios of proteins I and III in immunoprecipitates are consistent with these proteins being portions of a heteropolymer which retains this character in TX100 or Zw314. If this is the case, is protein I, protein III, or both the target(s) for antibodies in the immune rabbit sera? Protein III species present in apparent identical subunit MW are all resistant to proteolysis by exogenous proteases (1) in all GC strains and intrastrain derivatives investigated to date. Preliminary experiments utilizing GC strains having different protein I species (but having similar protein III species) demonstrate specific recognition, definite patterns of cross-reactivity, etc., by immunoprecipitation (unpublished observations); these findings argue for protein I being the dominant antigen since any appreciable immunogenicity for protein III would lead to marked cross-

reactivities among GC from diverse strains.

Finding that protein II is immunogenic and antigenic is of interest for several reasons. Previous studies by McDade and Johnston (16) showed that their protein 2 was not recognized by antisera raised against outer membrane fragments or "serotype-specific vesicles." Whether protein II is antigenic is important for interpretation of results such as those of Johnston et al. (11) in which outer membrane preparations from different strains may contain qualitatively and quantitatively different protein II moieties in addition to protein I constituents.

The immunogenicity, antigenicity, and surface exposure of protein II constituents are of interest also because the presence of these proteins on GC appears to vary depending on several host factors, and GC inhabiting an individual female host seem able to change their colonial opacity characteristics which are correlated with presence or absence of protein II constituents (4, 9, 10). Also, recent studies by Heckels and co-workers (7, 14) support those from my laboratory in showing that multiple forms of protein II can be found on GC of a single strain. The degree to which various protein II species are antigenically related is not known.

Several ^{125}I-labeled components other than proteins I, II, and III were found in immunoprecipitates. One of these (44K) is quite prominent in reactions with several rabbit antisera and may be an important contributor to the antigenic mosaic that comprises the GC surface.

Observations on the electrophoretic behavior of protein II species extracted with detergents are of interest in reference to heat-modifiable characteristics of these proteins. When boiled in SDS before SDS-PAGE, protein II species exhibit higher apparent subunit MWs than when they are solubilized at lower temperatures (55°C in the present study). The lower-MW form is identical with those found for protein II species extracted by TX100 or Zw314 even though these detergent lysates were boiled with 1% SDS before SDS-PAGE. This observation suggests that, under the conditions used here, SDS cannot displace TX100 or Zw314 molecules bound to protein II. This interpretation is consistent with previous authors' notions regarding the higher-MW form of the heat-modifiable *E. coli ompA* being the result of "unfolding" of the molecule when completely covered with SDS molecules (3, 17). Ethanol precipitation of the zwitterionic or nonionic detergent extracts probably removes these alcohol-soluble detergents from outer membrane protein-detergent micelles. Protein II, freed of zwitterionic ionic or nonionic detergent molecules, can fully bind SDS at boiling temperature and exhibit the higher-apparent-MW-band in SDS-PAGE.

The use of protein A covalently coupled to Sepharose beads was necessary in the current studies because of apparent interactions between GC proteins (predominantly protein II) and staphylococcal surface constituent(s). This interaction was prominent for protein II of GC outer membrane and staphylococci regardless of their having or lacking protein A; it was also independent of antiserum addition.

Differences in the apparent ratios of anti-protein I and anti-protein II antibodies in sera during the course of immunization (as in Fig. 7) and in sera derived from different animals inoculated with the same preparations of GC (as in Fig. 9) are somewhat analogous to the findings of Poolman and Zanen for human sera and antibodies directed against meningococcal outer membrane proteins (21). Those authors utilized different methods, but came to the conclusion that antibody specificities in patients' sera change over the course of meningococcal infections much the same as was seen here in rabbits sampled serially during their immunization with whole GC.

Several recent studies have utilized immunoprecipitation techniques to probe for the presence of antigenic constituents in gram-negative bacteria, such as *E. coli* K-12 (20) and yersiniae (22). In those studies, detergent lysis of outer membranes was followed by incubation with antiserum and subsequent immunoprecipitation by staphylococci. That experimental design yields little information regarding which antigenic constituents are exposed on the bacterial surface. A recent study on immunoprecipitation of gonococcal outer membrane proteins utilized outer membrane fragments which were reacted with antiserum lysed by a combination of anionic and nonionic detergents and immune complexes precipitated by protein A-bearing staphylococci (16). Techniques similar to those incorporated into the present study were used in a recently published investigation of surface antigens on *Trypanosoma cruzi* (19). It appears that this methodology may be useful for determining which antigens are exposed on microbial surfaces, cross-reactivities of various antigens from similar or diverse bacterial species, etc.

ACKNOWLEDGMENTS

My thanks to the following people for their contributions: Susan Smaus for preparation of this manuscript; Chuck Taylor and Bob Evans for art work; Osmar Barrera for antiserum preparations; and LMSF staff and Bruce Chesebro for many helpful discussions and manuscript review.

LITERATURE CITED

1. **Blake, M. S., E. C. Gotschlich, and J. Swanson.** 1981. Effect of proteolytic enzymes on the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **34**: 212-222.

2. **Buchanan, T. M., and W. A. Pearce.** 1979. Pathogenic aspects of outer membrane components of gram-negative bacteria, p. 475–514. *In* M. Inouye (ed.), Bacterial outer membranes. John Wiley & Sons, New York.
3. **DiRienzo, J. M., K. Nakamura, and M. Inouye.** 1978. The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions. Annu. Rev. Biochem. **47**:481–531.
4. **Draper, D. L., J. F. James, G. F. Brooks, and R. L. Sweet.** 1980. Comparison of virulence markers of peritoneal and fallopian tube isolates with endocervical *Neisseria gonorrhoeae* isolates from women with acute salpingitis. Infect. Immun. **27**:882–888.
5. **Dulbecco, R., and M. Vogt.** 1954. Plaque formation and isolation of pure lines of poliomyelitis virus. J. Exp. Med. **99**:167–182.
6. **Gotschlich, E. C.** 1980. The Neisseria, p. 642. *In* B. D. Davis, R. Dulbecco, H. N. Eisen, and H. S. Ginsberg (ed.), Microbiology. Harper & Row, Publishers, New York.
7. **Heckels, J. E.** 1977. The surface properties of *Neisseria gonorrhoeae*: isolation of the major components of the outer membrane. J. Gen. Microbiol. **99**:333–341.
8. **Heckels, J. E., and J. S. Everson.** 1978. The isolation of a new outer membrane protein from the parent strain of *Neisseria gonorrhoeae* P9. J. Gen. Microbiol. **106**: 179–182.
9. **James, J. F., C. J. Lammel, D. L. Draper, and G. F. Brooks.** 1980. Attachment of *N. gonorrhoeae* colony phenotype variants to eucaryotic cells and tissues, p. 213–216. *In* D. Danielsson and S. Normark (ed.), Genetics and immunobiology of pathogenic neisseria. University of Umea, Sweden.
10. **James, J. F., and J. Swanson.** 1978. Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. Infect. Immun. **19**: 332–340.
11. **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**: 741–758.
12. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lambden, P. R., J. E. Heckels, L. T. James, and P. J. Watt.** 1979. Variation in surface protein composition associated with virulence properties in opacity types of *Neisseria gonorrhoeae*. J. Gen. Microbiol. **114**:305–312.
15. **Markwell, M. A. K., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochemistry **17**:4807–4817.
16. **McDade, R. L., Jr., and K. H. Johnston.** 1980. Characterization of serologically dominant outer membrane proteins of *Neisseria gonorrhoeae*. J. Bacteriol. **141**: 1183–1191.
17. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *E. coli* K12. J. Biochem. (Tokyo) **80**:1411–1422.
18. **Newhall, W. J., W. D. Sawyer, and R. A. Haak.** 1980. Cross-linking analysis of the outer membrane proteins of *Neisseria gonorrhoeae*. Infect. Immun. **28**:785–791.
19. **Nogueira, N., S. Chaplan, J. D. Tydings, J. Unkeless, and Z. Cohn.** 1981. *Trypanosoma cruzi*: surface antigens of blood and culture forms. J. Exp. Med. **153**:629–639.
20. **Overbeeke, N., G. Van Scharrenburg, and B. Lugtenberg.** 1980. Antigenic relationships between pore proteins of *Escherichia coli* K12. Eur. J. Biochem. **110**: 247–254.
21. **Poolman, J. T., and H. C. Zanen.** 1980. Detection of antibody activity in human sera against meningococcal cell wall antigens using a gel-immuno-radioassay (GIRA), p. 107–116. *In* D. Danielsson and S. Normark (ed.), Genetics and immunobiology of pathogenic neisseria. University of Umea, Sweden.
22. **Straley, S. C., and R. R. Brubaker.** 1981. Cytoplasmic and membrane proteins of yersiniae cultivated under conditions simulating mammalian intracellular environment. Proc. Natl. Acad. Sci. U.S.A. **78**:1224–1228.
23. **Swanson, J.** 1978. Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infect. Immun. **19**:320–331.
24. **Swanson, J.** 1978. Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of *Neisseria gonorrhoeae*. Infect. Immun. **21**:292–302.
25. **Swanson, J.** 1979. Studies on gonococcus infection. XVIII. ^{125}I-labeled peptide mapping of the major protein of the gonococcal cell wall outer membrane. Infect. Immun. **23**:799–810.
26. **Swanson, J.** 1980. ^{125}I-labeled peptide mapping of some heat-modifiable proteins of the gonococcal outer membrane. Infect. Immun. **28**:54–64.
27. **Swanson, J.** 1980. Adhesion and entry of bacteria into cells: a model of the pathogenesis of gonorrhea, p. 17–40. *In* H. Smith, J. J. Skehel, and M. J. Turner (ed.), Dahlem workshop report on the molecular basis of microbial pathogenicity. Verlag Chemie GmbH, Weinheim, Germany.
28. **Swanson, J.** 1980. Gonococcal growth inhibition and hydrolysis of outer membrane proteins by alpha-chymotrypsin, p. 19–24. *In* D. Danielsson and S. Normark (ed.), Genetics and immunobiology of pathogenic neisseria. University of Umea, Sweden.
29. **Swanson, J., and J. Heckels.** 1980. Proposal: nomenclature of gonococcal outer membrane proteins, p. xxi. *In* D. Danielsson and S. Normark (ed.), Genetics and immunobiology of pathogenic neisseria. University of Umea, Sweden.
30. **Virji, M., and J. S. Everson.** 1981. Comparative virulence of opacity variants of *Neisseria gonorrhoeae* strain P9. Infect. Immun. **31**:965–970.