CHARACTERIZATION AND SPECIFICITY OF ANTIBODIES TO PROTEIN I OF *NEISSERIA GONORRHOEAE* PRODUCED BY INJECTION WITH VARIOUS PROTEIN I-ADJUVANT PREPARATIONS

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Our laboratory has been interested over the past several years in producing a safe and effective gonococcal vaccine. We have focused our attention on the use of purified gonococcal components to avoid the inherent toxicity of lipooligosaccharides (LOS)[1] present in vaccines using boiled organisms (1) or gonococcal membrane vesicles (2, 3) and to allow consistent production and effective standardization of the vaccine. The use of protein I (PI), the gonoccal porin (4–6), as a vaccine candidate appears to have several advantages.

PI, the major outer membrane protein of the gonococcus, is surface exposed (7), and its structure has been defined (8, 9). There are two main forms, PIA and PIB (10–12), and these can be further subdivided into serovars (13–15). PIA and PIB are structurally similar (8, 9) except for distinct areas that account for their antigenic and functional differences (7, 10, 14–21). PIA is present on gonococci that are resistant to complement-mediated killing by pooled normal human sera, and PIB is present on gonococci that are sensitive to killing by normal human sera (16, 17, 19, 20, 22). PI is antigenically conserved as opposed to pili, which seem to have infinite variability (23–25). Gonococcal protein III (PIII) is highly conserved as compared with PI but antibodies to PIII have been shown to block human serum killing of the organism (26–29). Antibodies elicited by PI have been demonstrated to be bactericidal (18, 21, 30) and opsonic (18, 21, 31). Additionally, PI does not undergo phase variations as seen with protein II (32–34).

Other investigators have used LPS-depleted membrane vesicles as a potential PI gonorrheal vaccine (2, 3). These vesicles were derived from whole organisms and therefore, in addition to PI, they contain other constituents of the gonococcal outer membrane that may be harmful or decrease the effectiveness of the anti-PI antibodies, e.g., by eliciting anti-PIII-blocking antibodies. Arminjon et al. (2) immunized

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[1] *Abbreviations used in this paper:* GB, gonococcal buffer; LOS, lipooligosaccharide; OG, octyl glucoside; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PI, protein I; PIII, protein III; PMN, polymorphonuclear cells; Z 3,14, Zwittergent *N*-tetradecyl-*N,N*-dimethyl-3-ammonia-propanesulfonate.

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subjects who had a history of previous gonococcal infections with such a vaccine. Many of the individuals' sera contained bactericidal and opsonic activity before inoculation, which decreased after immunization. Studies are being conducted presently to determine if this was a result of the production of PIII antibodies evoked by these vesicles because, as Rice et al. (26–28) have demonstrated, anti-PIII antibodies block human serum bactericidal killing of gonococci (29).

To optimize an effective antibody response to PI and reduce the potential production of anti-PIII-blocking antibodies, we chromatographically purified PI and then examined the immune response to this PI preparation combined with two different adjuvants. We investigated the effect of alum because its potentiation of the immune response has been shown for many antigens and it is currently the only adjuvant approved for human use. Liposomes were also studied because of their known adjuvant effects (35–40) and because we had previously shown that PI maintained both its structural conformation and function within these vesicles (6). Moreover, other investigators have used liposomes as carriers for several candidate vaccines as reviewed by Alving and coworkers (40, 41). Humans have tolerated liposome administration when utilized as drug carriers for various antibacterial, antiviral, or antifungal agents as recently reviewed by Popescu et al. (42) and Lopez-Berenstein and Juliano (43). Cancer chemotherapy drugs have also been administered utilizing liposomes as recently reviewed by Weinstein (44).

In this study different mixtures of PI combined with alum, liposomes, or Freund's adjuvant were prepared and injected into rabbits. The sera were examined for anti-protein I antibodies, and the specificity of these antibodies was analyzed, including the ability of these antibodies to be bactericidal and/or opsonic.

Materials and Methods

Organisms

The organisms used were a nonpiliated, transparent, serum-sensitive gonococcal strain originally isolated from a urogenital infection, Pgh 3-2 (kindly provided by Dr. Charles Brinton, University of Pittsburgh, Pittsburgh, PA), and a nonpiliated, transparent, serum-resistant gonococcal strain originally isolated from a disseminated infection, UU1 (kindly provided by Dr. Zell McGee, University of Utah, Salt Lake City, UT). Unless otherwise mentioned the isolates were used after 16–20 h of growth on gonococcal agar (45) in a 5% CO_2 incubator maintained at 37°C.

PI Isolation

PI was isolated by methods previously described (46–48). All purification steps were carried out at room temperature unless stated otherwise. The bacteria were harvested by centrifugation and slowly resuspended in an equal volume of 1.0 M sodium acetate, pH 4.0, containing 1 mM 2,3-dimercaptopropanol. To this suspension was added 6 vol of 5% (wt/vol) Zwittergent *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonia-propanesulfonate (Z 3,14) (Calbiochem-Behring Corp., La Jolla, CA) in 0.5 M $CaCl_2$, and this was stirred for 1 h. 2 vol of absolute ethanol was added slowly to bring the concentration to 20% (vol/vol). The precipitate, which contained most of the nucleic acids, was removed by centrifugation at 17,000 *g* for 15 min. The concentration of ethanol in the supernate was increased to 80% (vol/vol) and the resultant precipitate was recovered by centrifugation. This precipitate, which contained PI, was resuspended in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 5% Z 3,14. This mixture was stirred for 1 h and clarified by centrifugation at 12,000 *g* for 15 min. The soluble material was applied to two columns (2.6 × 30 cm) linked in tandem, one packed with DEAE Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) and the second packed with CM Sepharose CL-6B (Pharmacia Fine Chemicals), both equilibrated with 50 mM Tris HCl, pH 8.0, with

10 mM EDTA and 0.05% Z 3,14. The eluate was monitored by 280 nm absorbance and SDS-PAGE. After sample application, the columns were washed with equilibration buffer until the 280 nm absorbance reached baseline. The majority of PI flowed through both columns. The flow through was saved and the PI was precipitated by the addition of absolute ethanol to a concentration of 80% (vol/vol). The precipitate was retrieved by centrifugation at 12,000 *g* for 15 min. This precipitate was dissolved in 10 ml of 50 mM Tris-HCl, pH 8.0, with 10 mM EDTA and 5% Z 3,14 and was applied to a column (2.6 × 170 cm) of Sephacryl S-300 (Pharmacia Fine Chemicals). The elution buffer was 100 mM Tris-HCl, 200 mM NaCl, 10 mM EDTA, 0.05% Z 3,14, pH 8.0, and the flow rate was 10 ml/h. Fractions of 7.5 ml were monitored for absorbance at 280 nm, collected, and analyzed by SDS-PAGE. The fractions that contained PI were pooled and utilized in the immunizing preparations.

PI-Adjuvant Preparations

Alum PI. The purified PI described above was filtered through a 0.22-μm filter (Millipore Continental Water Systems, Bedford, MA) with no significant loss of the protein as measured by Coomassie blue assay (49). All further steps were done under aseptic conditions. To determine the maximum amount of PI that could be bound to alum, aluminum hydroxide (Alu-Gel-S; Serva Feinbiochemica, Heidelberg, FRG) was washed with 0.9% NaCl and aliquoted. PI was added in increasing amounts to a series of identical alum aliquots, mixed, and rotated for 1 h at room temperature. These samples were centrifuged and the supernates were removed carefully. The amount of PI that remained unabsorbed was determined by the Coomassie blue assay (49). Up to 1.5 mg of PI could bind to 10 mg of alum. Various amounts of PIB were bound to 10 mg of alum to obtain different PI/alum ratios: 1:10 (1,000 μg PI), 1:40 (250 μg PI), 1:100 (100 μg PI). For the PIA experiments 200 μg PI was bound to 10 mg of Alum. After repeated 0.9% NaCl washes, the 10 mg alum PI mixtures were resuspended in 1 ml of 0.9% NaCl.

Liposome PI. Liposomes were prepared by a variation of a described method (50) as follows. (*a*) The detergent in which the PI was dissolved was changed to D-octyl glucoside (OG) (Aldrich Chemical Co., Milwaukee, WI) by precipitating the PI by the addition of ethanol, 50 mM sodium acetate, to a concentration of 66.7% (vol/vol), centrifuging this mixture at 10,000 *g* for 10 min, and resuspending the precipitate in 10% (wt/vol) OG in 10 mM Hepes; (*b*) the liposomal lipids, synthetic phosphatidyl ethanolamine (PE), and phosphatidyl choline (PC) (Avanti Polar Lipids, Inc., Birmingham, AL) dissolved in chloroform (20 mg/ml), were mixed in a ratio of 3:1 (100 mg PE/33 mg PC) for PIB and 4:1 (16 mg PE/4 mg PC) for PIA and dried in a thin layer on the inside of an acid-cleaned test tube by rotary evaporation; (*c*) 3 ml of purified PIB, 1 mg/ml, or 1 ml of purified PIA, 4 mg/ml, in the OG was added to the dried PC/PE mixture, which dissolved the lipids and gave a clear solution; (*d*) the solution was dialyzed extensively against PBS, pH 7.2, to remove the OG and induce liposome formation; and (*e*) the dialyzed solution was sonicated (Branson Sonic Water Bath, Plainview, NY) to form small unilammellar vesicles. EM confirmed the production of small unilammellar vesicles (kindly done by Drs. D. Dorset and A. Mikalski, Buffalo, NY). To determine if the PIB is oriented in the liposomes in a similar way to that observed in the intact outer membranes, the chymotryptic-generated peptides of PIB in liposomes, purified PIB in detergent, and PIB associated with intact bacterium were compared. Samples of each were prepared such that each had similar quantities of PIB (1 mg/ml). Chymotrypsin (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to each sample in a PIB to enzyme ratio of 25:1 (mg/mg) and incubated for 30 min at 37°C. The samples were then analyzed by SDS-PAGE. Liposomes containing PIA were analyzed in a similar way, using proteinase K (Sigma Chemical Co., St. Louis, MO) as the proteolytic enzyme.

Freund's Adjuvant PI. The primary injections were made by emulsifying 0.2–0.5 mg of PI with 1.0 ml of CFA (Difco Laboratories Inc., Detroit, MI). For all subsequent injections, the emulsion was made using IFA.

Immunization Protocol

New Zealand white rabbits (Marland, Hewitt, NJ) weighing 2.5–3.0 kg were used in all experiments. Rabbits were immunized subcutaneously with the various PI preparations. For timing and amounts see Table I.

Whole Organism Antisera Production

10^8 CFU of Pgh 3-2 or UUI mixed with CFA (Difco Laboratories, Inc.) was injected into a rabbit, and 3 wk later the rabbit was boosted with the same amount of organisms in IFA.

ELISA Determinations

Microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were sensitized by adding 0.1 ml per well of PI (2 μg/ml), in 0.05 M sodium carbonate buffer, pH 9.8, with 0.02% azide. The plates were incubated overnight at room temperature. The plates were washed five times with 0.9% NaCl, 0.05% Brij 35, 10 mM sodium acetate, pH 7.0, 0.02% azide. The rabbit sera, starting at a dilution of 1:1,000 in 10 mM Tris, pH 8.2, 0.15 M NaCl, 0.05% Brij 35, 0.02% azide (incubating buffer), were incubated on the plate for 4 h at room temperature. The plates were again washed as before and the appropriate secondary antibody, either alkaline phosphatase-conjugated goat anti-rabbit (Tago Inc., Burlingame, CA) or goat anti-mouse (Tago Inc.), were diluted in the incubating buffer, added to the plates, and incubated for 2 h at room temperature. The plates were washed as before and *p*-nitrophenyl phosphate (Sigma Phosphatase Substrate 104; Sigma Chemical Co.) (1 mg/ml) in 0.1 M diethanolamine, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.02% azide, pH 9.8, was added. The plates were incubated at 37°C for 1 h and the absorbance at 405 nm was determined using an Elida-5 spectrophotomer (Physica, New York, NY). Control wells lacked either the primary and/or secondary antibody. To determine the amount of antibody raised to a known surface-exposed area on PIB, a synthetic peptide containing the amino acid sequence ser-ile-pro-ser-leu-phe-val-glu-lys-leu-gln-val-his-arg-leu-(cys) was used. This sequence is found in PIB at amino acid residues 199–213 and starts at the chymotrypsin cleavage site in this molecule. The microtiter plates were sensitized as before with a peptide concentration of 0.5 μg/ml. The ELISA values are reported as the dilutions that gave an absorbance of 1.0 at 405 nm after a 1-h incubation.

SDS-PAGE and Western Blot

The SDS-PAGE was a variation of Laemmli's method (51) as described previously (47). Electrophoretic transfer to nitrocellulose was performed according to the methods of Towbin et al. (52) and the Western blots were probed with phosphatase-conjugated reagents (53). Primary and secondary antibodies were the same as those used in the ELISA assays described above.

Absorption of Sera with Whole Organisms

Gonococci were harvested from agar plates with a dacron swab and resuspended in a Proteose Peptone No. 3 broth (Difco Laboratories, Inc.) with 0.9% NaCl. The turbidity was measured on a spectrophotometer (Perkin-Elmer Corp., Maywood, IL) set at 600 nm and the cells were diluted to an absorbance of 0.1 ($\sim 10^8$ CFU/ml). The suspended organisms were aliquoted so that when collected by centrifugation and resuspended in diluted antisera, the final concentration of organisms was $\sim 10^8$/ml. The various sera were titrated in 0.9% NaCl to a dilution that gave an ELISA value of 1.0. The diluted sera were added to the centrifuged organisms, the organisms were resuspended, and the mixtures were incubated on ice for 30 min. The bacteria were concentrated by centrifugation and the serum was carefully removed. After each absorption, a small volume from each aliquot of absorbed serum was removed for ELISA analysis. Continued absorptions were done a total of five times. The ELISA values of absorbed sera were compared with that of unabsorbed sera at the same dilution.

Microtiter Bactericidal Assay

A method similar to that described by Schneider and Griffiss (54) was used to determine the bactericidal activity of antisera. They were diluted 1:40 and then serially diluted twofold in microtiter plates (Microtest III; Becton Dickinson & Co., Mountain View, CA). Organisms, grown for 16–20 h on gonococcal agar were resuspended in gonococcal buffer (GB, 0.01 M potassium phosphate buffer, pH 7.2, containing NaCl 7.94 g/liter, KCl 0.37 g/liter, $CaCl_2$-2X H_2O 0.25 g/liter, $MgCl_2$-$6H_2O$ 0.06 g/liter, $MgSO_4$-$7H_2O$ 0.246 g/liter, BSA 0.01%) and diluted to an OD of 0.1 at 600 nm ($\sim 10^8$ CFU/ml). Reaction mixtures in each well consisted

of 25 µl of heat-inactivated diluted sera, 10 µl hypogammaglobulinemic serum as a complement source (kindly provided by Dr. Robert Lahita, The Rockefeller University, New York, NY), 25 µl of gonococci, and 40 µl of GB. The antiserum dilutions were done in duplicate and control wells lacked added complement. The microtiter plates were incubated for 30 min at 37°C. Then 100 µl of gonococcal media (45) with low melting point agarose (FMC, Rockland, ME) cooled to 37°C was added to each well and the entire plate incubated in a 37°C, 5% CO_2 incubator for 24 h. The bactericidal titer was considered to be the dilution in the last well that had >95% killing.

Agglutination of Whole Organisms

Sera were diluted 1:10 in GB and 0.1 ml was added to 0.1 ml of log phase whole organisms diluted to an OD of 0.1 at 600 nm. Agglutination was graded by light microscopy on a scale from 0 to 4+.

Opsonophagocytosis Assay

To measure the opsonic effects of the sera, a variation of the procedure described by Ross et al. (55) was used. Polymorphonuclear cells (PMN) were procured by collecting whole blood of normal human volunteers in a heparinized syringe. The blood was diluted 1:1 with sterile 2% gelatin in 0.9% NaCl and was allowed to stand for 30 min at 37°C. The serum fraction was removed and centrifuged at 3,000 *g* for 15 min. The residual RBC in the PMN-enriched pellet were lysed by the addition of 0.85% NH_4Cl, and the PMN were recovered by centrifugation. PMN were washed twice in M199 (KC Biological, Inc., Lenexa, KS) and resuspended at a concentration of 10^7 PMN/ml. Viability of the PMN was assessed by the trypan blue exclusion assay. The gonococci were grown to exponential phase in Proteose Peptone no. 3 (Difco Laboratories, Inc.) broth with defined supplements (Isovitalex; BBL Microbiology Systems, Cockeysville, MD). The organisms were washed with GB and resuspended to an OD of 0.1 at 600 nm ($\sim 10^8$ CFU/ml) and diluted 10-fold. The volume of the reaction mixture was 0.25 ml, which consisted of 10% undiluted antiserum, 10% undiluted C8-deficient serum (kindly provided by Dr. Keith Joiner, NIH), 0.1 ml of the diluted organisms ($\sim 10^6$ CFU), and 0.1 ml of the resuspended PMN ($\sim 10^6$). Each reaction mixture was put in a 12 × 75-mm snap cap plastic tube (Becton Dickinson Labware, Oxnard, CA) and rotated for 30 min at 37°C. Samples at zero time and 30 min were taken and diluted appropriately to measure survival. To test the opsonizing ability of mAb 9A1, an mAb to PIB, 25 µl of undiluted mAb was added to the reaction mixture instead of immune sera. The relative survival was the ratio of CFU in the reaction mixtures containing C8D serum to the reaction mixtures without C8D. Controls were mixtures without antiserum and/or C8D.

In some cases, the sera of rabbits displayed opsonic activity before immunization. Unfortunately, this was determined only after these rabbits had been inoculated several times with a PIA liposome preparation. To demonstrate that the opsonizing antibodies in these immune sera were to PIA and not to other proteins (see Fig. 6), the PIA-reactive antibodies were absorbed from one rabbit's serum, whose preimmune serum was not opsonic, on a PIA affinity column until there was minimal PIA reactivity as demonstrated by Western blot (data not shown). This absorbed serum was tested in the opsonophagocytic assay described above and the relative survival of UU1 utilizing PIA-absorbed serum was compared with the unabsorbed immune serum and its preimmune counterpart.

Results

The purpose of this study was to evaluate several different immunization methods to evoke functional antibodies to an integral membrane protein, i.e., the porins of *Nesseria gonorrhoeae*. The functional aspects of these antibodies were defined as their ability to (*a*) aggregate whole organisms; (*b*) be bactericidal; and (*c*) be opsonic for gonococci. We used purified PI in FCA, bound PI to alum phosphate, and incorporated PI into liposomes. Of these three methods used to prepare PI for immuniza-

tion, the last two have been approved for human use. The immunization protocol of each of the preparations is shown in Table I.

To demonstrate that PI when inserted into liposomes has a similar orientation as in the intact organisms, the PIA and PIB liposomes were treated with proteinase K or chymotrypsin, respectively. The resulting proteolytic fragments of both PIB and PIA in liposomes migrated in SDS-PAGE like those generated when these enzymes were added to intact gonococci expressing these proteins (Figs. 1 and 2) (7).

After three immunizations with PIB, the immune rabbit sera evoked by each of the preparations gave comparable ELISA values with the exception of FCA, which elicited a 10-fold higher response in this assay (Table II). Furthermore, the Western Blot analysis indicated that the antibodies were reactive and specific for PIB (Fig. 3). However, in comparing these immune sera for their ability to agglutinate intact gonococci, they differed quite radically. The immune sera raised using the alum preparations agglutinated Pgh 3-2 very weakly (0-1+) and at lower titers. Whereas, the antisera raised using liposomes agglutinated Pgh 3-2 more avidly (2-3+) and at higher dilutions. This suggested to us that more of the antibodies in the immune sera produced by the liposomes were directed at surface-oriented epitopes on the gonococci. Further evidence for this was seen in the assays measuring the bactericidal titers (Table II) and opsonizing ability (Table III) of each of the antisera. As can be seen, the immune antisera raised with the liposomes were between two- and eightfold more bactericidal than those raised with alum and were equivalent to the immune sera elicited with Freund's adjuvant. The PI liposome immune sera also showed greater opsonophagocytosis activity compared with the alum sera (Table III).

Similar experiments were performed using purified PIA from strain UU1. The ELISA values of the immune sera raised to PIA using alum as an adjuvant were comparable with those seen with the PIB (Table II). But the titers of the antisera from rabbits inoculated with PIA liposomes were 5-10-fold lower in this assay. However, as with the PIB experiments, the PIA liposome antisera had two- to fivefold higher bactericidal titers than the PIA alum antisera (Table II) despite their lower ELISA values. The Western blots of the PIA antisera demonstrated their reactivity to PIA (Fig. 4) but also showed reactivity with other gonococcal proteins, including PIII. To demonstrate that it was the PIA antibodies that were opsonic, PIA-liposome

TABLE I

Immunization Protocol

Organism	Adjuvant	Amount PI	Injections	Interval	Rabbits
		mg		*wk*	
Pgh 3-2	Alum	1.0	3	3	1
		0.25	3	3	1
		0.10	3	3	1
	Liposome	0.10	3	1	1
		0.20	3	1	1
	Freund's	0.20	2	3	1
UU1	Alum	0.20	3	2	2
	Liposome	0.20	3	2	2
	Freund's	0.20	2	2	1

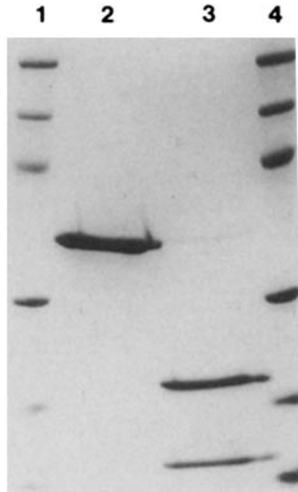

FIGURE 1. SDS-PAGE gel of PIB liposomes. Lanes *1* and *4*, molecular weight standards; lane *2*, PIB liposome, 25 μg PIB, untreated; lane *3*, PIB liposome, 25 μg PIB, treated with 1 μg chymotrypsin at 37°C for 30 min (51). There was almost total degradation of PIB by chymotrypsin demonstrating that a high percentage of the chymotrypsin sensitive site of PIB exposed in vivo is also exposed in the liposome construct (7). Molecular weight standards were human transferrin (80,000), catalase (60,000), OVA (45,000), carbonic anhydrase (30,000), soy bean trypsin inhibitor (21,500), and cytochrome *c* (12,500).

immune serum, from a rabbit whose preimmune serum was not opsonic, was absorbed with purified PIA to remove the PIA antibodies. This serum was then reassessed for its reactivity by Western blot analysis and the opsonophagocytic assay. The Western blot demonstrated that most if not all the antibodies reactive to PIA had been removed while the reactivity to the other gonococcal proteins remained (data not shown). The serum exhibited a marked loss of opsonic activity to UU1 gonococci (as compared with the antiserum before absorption), percent survival was 81% vs. 0%, respectively.

Both of these studies pointed to the fact that a higher percentage of the antibodies elicited by PI incorporated into liposomes were directed at surface epitopes on the gonococci. Two methods were used to confirm this hypothesis. A synthetic peptide was made that extended from the know surface-exposed chymotrypsin cleavage site consisting of amino acid residues 119–214 in the PIB sequence (56). Using this peptide in an ELISA assay, the PIB liposome-generated antibodies were compared with

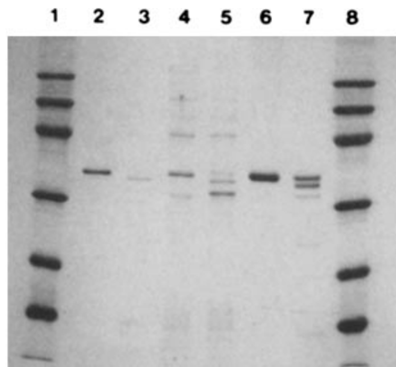

FIGURE 2. SDS-PAGE of PIA liposomes. Lanes *1* and *8*, molecular weight standards as in Fig. 1; lane *2*, purified PIA, 25 μg, untreated; lane *3*, purified PIA, 25 μg, treated with proteinase K, 1 μg; lane *4*, whole UU1 organism, untreated; lane *5*, whole UUI organism treated with proteinase K; lane *6*, PIA liposome, untreated; lane *7*, PIA liposome treated with proteinase K.

TABLE II
Serum Bactericidal Titers

Antisera	Bactericidal titer	PI ELISA titer
Pgh 3-2		
Preimmune	1:320	0
Alum 1:10	1:640	1/250,000
Alum 1:40	1:640	1/240,000
Liposome (0.10 mg)	1:1,280	1/220,000
Liposome (0.20 mg)	1:5,120	1/180,000
Freund's	1:5,120	1/2,400,000
Whole organism	1:20,480	1/230,000
UU1		
Preimmune	1:1,280	0
Alum no. 1	1:1,280	1/200,000
Alum no. 2	1:1,280	1/94,000
Liposome no. 1	1:5,120	1/24,000
Liposome no. 2	1:2,560	1/24,000
Freund's	1:1,280	1/270,000

the PIB alum-generated antibodies for reactivity to this area. As can be seen in Fig. 5, the ELISA values for the liposome-elicited antibodies were much higher in this assay (1:2,800 vs. 1:870). The various PIB antisera were serially absorbed with intact Pgh 3-2; the antisera raised utilizing PIB liposomes retained <45% of their reactivity, while the antisera raised utilizing PIB alum retained >75% of their reactivity after absorption (Fig. 6).

Discussion

The major outer membrane protein of the gonococcus is PI and comprises up to 60% of the membrane protein of the organism (46). PI has distinct advantages

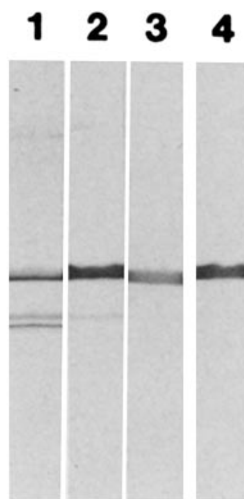

FIGURE 3. Pgh 3-2 Western Blot. Whole cell lysate of strain Pgh 3-2 was transferred to the nitrocellulose. Lane *1*, PIB liposome antiserum 0.100 mg PIB; lane *2*, PIB Alum antiserum, 1:10 PIB to alum ratio, 10 mg alum; lane *3*, PIB Freund's antiserum; lane *4*, PIB mAb 9A1.

TABLE III
Pgh 3-2 (PIB) Opsonophagocytosis Survival

Antisera	Relative survival
None	0.65
Preimmune	0.67
mAb 9A1	0.05
Alum PI 1:10	1.39
Alum PI 1:40	1.04
Liposome PI (100 μg PI)	0.05
Liposome PI (200 μg PI)	0.04
Freund's PI	0.07

Reaction mixture volume was 0.25 ml containing 10% undiluted sera, 10% C8-deficient sera (kindly provided by Dr. Keith Joiner, NIH), 0.1 ml of PMNs (10^7 PMNs/ml), and 0.1 ml of diluted organisms (~10^7 CFU/ml). Reaction mixtures were rotated for 30 min at 37°C and samples at zero time and 30 min were taken and diluted appropriately. C8D did not contain bactericidal activity. Relative survival was a ratio percent survival of CFU at 30 min in C8D plus tubes vs. C8D minus tubes (55).

as a gonococcal vaccine candidate. PI is antigenically conserved (14, 15) and portions of PI are exposed on the surface of the organism (7). It has been demonstrated by Joiner et al. (30) adn Virji et al. (18, 21) that antibodies produced to these surface-exposed epitopes are bactericidal, opsonic, and can protect against gonococcal invasion in an in vitro cell culture invasion assay. Furthermore, Sarafian et al. (31) demonstrated Fab portions of PI mAb can block opsonic activity of human sera.

As stated, other investigators have used LPS-depleted membrane vesicles as a potential PI antigonococcal vaccine (2, 3). These vesicles in addition to PI inevitably con-

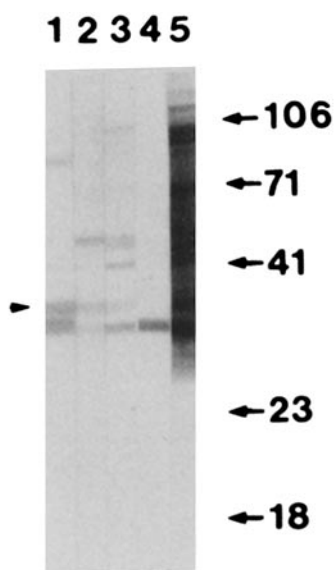

FIGURE 4. UU1 Western blot. Whole cell lysate of strain UU1 was transferred to nitrocellulose. Lane *1*, PIA alum antisera, 1:1,000; lane *2*, PIA liposome antisera 1:1,000; lane *3*, PIA Freund's antisera, 1:1,000; lane *4*, PIII antisera; lane *5*, India ink. Arrowhead points to PIA band.

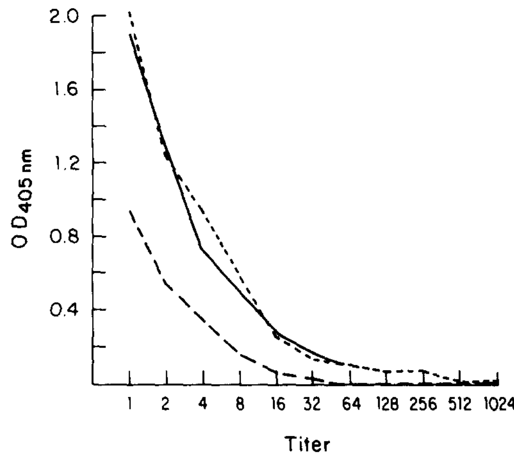

FIGURE 5. ELISA titers of antisera to PIB surface oligopeptide. Oligopeptide used (ser-ile-pro-ser-leu-phe-val-glu-lys-leu-gln-val-his-arg-leu-[cys]) is equivalent to the NH_2 terminus of the chymotrypsin-cleaved portion of PIB, which is surface exposed. (*Abscissa*) Reciprocal of the dilution × 1,000; (*ordinate*) OD_{405} of dilution. Intercept of OD_{405} at 1.0 (half-maximal OD) is titer of that sera used. PIB alum (— — —) PIB liposome (---------), PIB Freund's (———).

tained some amount of PIII. A potential problem with the presence of PIII is the induction of anti-PIII-blocking antibodies (26–29). In the recent PI vaccine trial by Arminjon et al. (2), multivalent gonoccocal vesicles, that contained PI and <10% PIII, were injected into volunteers with a history of previous gonococcal infections. After immunization, the bactericidal and opsonic ability of the subjects' sera against the gonococcus decreased instead of increasing as expected. The production of anti-PIII-blocking antibodies is presumed to be the reason for this finding and further studies are under way to confirm this hypothesis. To avoid these complications, we wanted to use detergent-solubilized, chromatographically purified PI with minimal PIII or LOS contamination.

Our next task was the presentation of the purified PI in a manner that would elicit the most effective antibodies and, therefore, the method of presenting this porin in the most natural way was carefully considered. Studies of *Escherichia coli* outer membrane porins (57, 58) demonstrate that they have a complex tertiary structure with a large portion of the molecule in β sheet conformations. These monomers com-

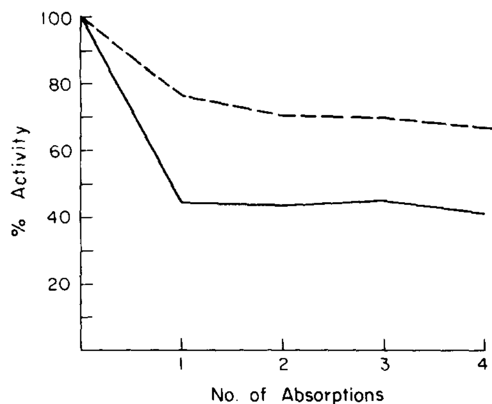

FIGURE 6. Whole cell absorptions. Sera were absorbed with whole Pgh 3-2 P-Tr. The sera were diluted to a titer that gave an OD_{405} of 1.0 by ELISA. All absorptions and dilutions were done in sterile 1% proteose peptone (Difco Laboratories, Inc.), 0.9% NaCl. Each milliliter of diluted sera was absorbed with ~10^8 CFU of whole organism (OD_{405}, 0.1). The absorptions were done on ice for 30 min. A small volume was saved from each absorption for the ELISA and sequential absorptions were performed for a total of five times. ELISA values relative to the unabsorbed diluted sera were determined. PI alum (— — —); PI liposome (———).

bine in a trimeric quaterinary structure. PI, the porin of *Neisseria gonorrhoeae*, likewise forms trimers (46) and is structurally related to the *E. coli* porins (8). Thus, these porins can be envisioned as three cylinders that are bound together and span the Gram-negative outer membrane. They have surface-exposed areas, regions that represent the internal part of the cylinder, and portions that extended into the periplasmic space. Antibodies elicited to other than surface-exposed epitopes would thus be ineffective as we have demonstrated.

In this study, purified PI was presented absorbed to alum or incorporated into liposomes. In each case a humoral immune response was induced and high anti-PI titers were detected in an ELISA assay. The antisera raised by both preparations recognized PI on western blots, but the alum PI-induced sera did not agglutinate intact gonococci and did not have an increase in bactericidal or opsonic activity. Furthermore, these antibodies appeared to react poorly with surface-exposed epitopes. On the other hand, PI inserted into liposomes in its native orientation as demonstrated by enzymatic studies (Figs. 1 and 2) raised antibodies that agglutinated intact organisms and the sera had higher bactericidal and opsonic activity than the preimmune sera and the PI alum sera. In addition the liposome antisera contained a higher percentage of antibodies to exposed epitopes of PI as demonstrated by the synthetic peptide ELISA assay and whole cell absorptions.

Other authors have shown that antigens associated with liposomes can be used as immunogens and that liposomes themselves can act as an adjuvant (35–38, 40–41) and liposomes themselves are well tolerated by humans (41–44). In this study, the liposome-generated sera had greater functional reactivity towards the gonococcus than the alum-generated sera. A possible reason that PI alum antisera produced a smaller percentage of antibodies to exposed epitopes of PI is that alum is ionic in nature. The alum may bind to the ionic, hydrophilic-exposed portions of the protein and mask these epitopes from the immune system. However, when PI is inserted in the liposome, the epitopes displayed in vivo are exposed on the liposome. Antibodies produced utilizing this method are thus to the surface-exposed portions of the protein. Jiskoot et al. (38) recently published a report where they raised antisera to PI utilizing alum, detergents, and liposomes in various combinations. They raised the greatest ELISA antibody titers to PI with alum, but they did not characterize the functional activity of the antisera.

Even though the PI preparations used as immunogens contained minimal PIII contamination (<1%), there was significant reactivity with PIII on the Western blot (Figs. 3 and 4). PIII is probably an excellent immunogen and can elicit a greater antibody response per quantity of protein than the other membrane proteins of the gonococcus. This might be helpful to the gonococcus especially since it appears that antibodies to PIII have blocking capabilities (26–29) and PIII is antigenically conserved (48, 59, 60). Despite the production of antibodies that recognized PIII on the Western blots, we still observed increased bactericidal and opsonic activity.

In conclusion, PI is an obvious choice for a gonococcal vaccine candidate. The adjuvant used to present PI as a vaccine can affect the epitopes of the protein that are exposed to the host immune system and, therefore, the specific epitopes antibodies raised will recognize. PI inserted into liposomes is a practical way to immunize against a gonococcal antigen and raise antibodies to its surface with minimal blocking antibody production. It is important to mention that we could not fully separate

all PIII from PI and, therefore, some PIII antibodies were raised but these antibodies did not appear to block bactericidal or opsonic activity.

Summary

A major goal of gonococcal research is the development of a gonorrheal vaccine. A vaccine candidate is the major outer membrane protein (PI) of the gonococcus, which has limited antigenic variability. Two main subtypes, PIA and PIB, and nine main serotypes have been described. To avoid raising anti-protein III (PIII)-blocking antibodies and limit potential lipooligosaccharide toxicity, PI was chromatographically isolated with minimal PIII contamination (<1%) from Pgh 3-2 (PIB), a serum-sensitive gonococcal strain and UU1 (PIA), a serum-resistant gonococcal strain. Alum was used as an adjuvant and the antibodies raised in rabbits did not agglutinate the organisms, were not opsonic, and bactericidal titers were not increased. To present PI in a form mimicking its in vivo disposition, it was inserted into liposomes. The resulting antisera did agglutinate the organism and contained opsonic and bactericidal activity greater than the preimmune sera or alum-generated sera. The PIB liposome antisera also had higher ELISA titers to a synthetic peptide equivalent to an exposed portion of PIB and a higher percentage of antibodies absorbed by whole organisms than the PIB alum antisera. We speculate that when PI is presented in liposomes, the antibodies raised are mainly to surface-exposed epitopes of the protein as opposed to when PI is presented absorbed to alum, where the antibodies are produced mainly to buried epitopes.

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