Surface-Exposed Antigenic Cleavage Fragments of Neisseria gonorrhoeae Proteins IA and IB

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Whole bacteria, isolated outer membranes, and purified protein I (PI) from one transparent (O−) and two different opaque (O+) phenotype gonococcal strains (serogroups I, II, and III; PI serotypes 1, 5, and 9b) were each treated with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin, alpha-chymotrypsin, and proteinase K. Protein IA (PIA) of strain 7122 (O−, serotype 1, serogroup I) was resistant to proteolysis by tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin and alpha-chymotrypsin and only slightly affected by proteinase K, as long as it was associated with intact bacteria or isolated outer membranes. Purified PIA however was cleaved by these enzymes, resulting in two to five fragments. In contrast, all preparations of strains 5766 opaque phenotype (O+, serotype 7, serogroup II) and 1955 (O+, serotype 9b, serogroup III) were accessible to proteolysis, resulting in cleavage fragments of PIB compatible to those described previously by O. Barrera and J. Swanson (Infect. Immun. 44:565–568, 1984). M. S. Blake et al. (Infect. Immun. 33:212–222, 1981), and Blake (in G. K. Schoolnik, ed., The Pathogenic Neisseriae, 1985). Our data indicated that the purified PIB fraction was more accessible to proteases than the PIBs of whole bacteria or outer membranes. The fragmentation pattern of PIA cleavage products were quite different from PIB fragments, consistent with the different structure of these two groups of PIB molecules. Time-dependent cleavage experiments with proteases, i.e., alpha-chymotrypsin, indicated that PIA was subsequently cleaved into smaller fragments. Highly reactive monoclonal antibodies, each specific for a surface-exposed epitope of PIA of strain 7122 or PIB of strains 5766 and 1955, as assessed by coagglutination, Western blot, and immunofluorescence, were reacted with PIA and PIB cleavage fragments in Western blot experiments. All cleavage fragments of the purified PIA and PIB preparations with molecular weights of ≈14,200 showed immune reaction in Western blotting, whereas whole cell and outer membrane PIB fragments were less reactive with the specific monoclonal antibodies.

Materials and Methods

Bacteria. The gonococcal strains utilized were 7122 (serotype 1, serogroup I), 5766 (serotype 7, serogroup II), and 1955 (serotype 9b, serogroup III) (6, 16). These gonococcal strains were nonpiliated, colony type 3 and 4 organisms as determined by the typing scheme of Kellogg et al. (10). Strain 7122 was of the transparent phenotype (O−), and both 5766 and 1955 were phenotypically opaque (O+). Bacteria were kept frozen at −70°C in freezing medium containing 5% bovine serum albumin and 5% monosodium glutamate and freshly grown on GC agar (Difco Laboratories, Detroit, Mich.) with added Kellogg supplement (22) at 37°C in 5% CO₂ for 19 to 21 h.

Chemicals and reagents. The following chemicals and reagents were used: tosyl lysine chloromethyl ketone (TPCK)-trypsin, TLCK-treated alpha chymotrypsin (both enzymes from Sigma Chemical Co., St. Louis, Mo.); proteinase K (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany); phenylmethylsulfonyl fluoride (Sigma); Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, N.J.); 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.); peroxidase-conjugated fraction of goat anti-mouse immunoglobulin G (IgG; Cooper Biomedical, Inc., West Chester, Pa.); and peroxide (30%; J. T. Baker Chemical Co., Phillipsburg, N.J.). All other chemicals were purchased from commercial sources.

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MAbs. Monoclonal antibodies (MAbs) were prepared as previously described (8, 19) by using an enzyme-linked immunosorbent assay to screen the fusion products. Each MAb was proven to be specific for protein I by Western blot and gel immunoradio assay. The MAbs chosen recognized only one of the three strains studied, respectively. These were NG2D12A (strain 7122, serotype 1), NG23E9F (strain 5766, serotype 7), and G14B3F (strain 1955, serotype 9b). All MAbs used in this study were strongly reactive with a surface-exposed epitope as assessed by coagglutination and immunofluorescence.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (0.75 and 1.5 mm) were poured with 12.5% acrylamide by the method of Laemmli (12) and with 20% acrylamide containing 8 M urea by the method of Kyte and Rodriquez (11).

Purification of PI. PI was purified as described by Poolman and Buchanan (15), with three ultracentrifugations at 150,000 \( \times g \) and two at 450,000 \( \times g \). The resulting pellet contained PI. The following steps were performed at 25°C. The pellet was suspended in 50 mM glycine buffer (pH 10.0) containing 0.15 M ethanolamine, 10 mM EDTA, 0.2 M NaCl, and 5% N-tetradecyl-N',N'-dimethyl-3-ammonio-1-propanesulfonate and applied to a Sepharose 6B column (100 by 2.6 cm).

Protein was eluted with 50 mM glycine buffer (pH 9.0) containing 0.17 M ethanolamine, 0.2 M NaCl, 10 mM EDTA, and 0.1% N-tetradecyl-N',N'-dimethyl-3-ammonio-1-propanesulfonate at a flow rate of 10 ml/h. Fractions of 5 ml were collected. The purity of PI was checked in SDS-PAGE. The PI fractions were pooled and dialyzed against 50 mM glycine, 50 mM ethanolamine, and 0.1% N-tetradecyl-N',N'-dimethyl-3-ammonio-1-propanesulfonate at pH 8.0.

Isolation of OMs. Gonococci were grown on solid medium and harvested after 19 to 21 h. The cells were suspended in 0.2 M Tris-hydrochloride buffer (pH 8.0) and sheared in a Trocellulose (Janke and Kunkel, IKA Werk, Federal Republic of Germany) at setting 30 without foaming for 15 min at 45°C. Homogenized bacteria were centrifuged at 12,000 \( \times g \) for 15 min at 4°C. The supernatant was removed and spun at 30,000 \( \times g \) for 20 min at 4°C. That supernatant was centrifuged again at 145,000 \( \times g \) for 90 min at the same temperature. The glassy pellet was suspended in saline, centrifuged for 90 min at 155,000 \( \times g \) (4°C), and solubilized in saline. The membrane protein concentration was adjusted to 1 mg/ml. The membrane solutions were aliquoted and frozen at -70°C.

LPS and protein determination. The total lipopolysaccharide (LPS) content was calculated by estimating that gonococcal LPS contained 8% (by weight) 2-keto-3-deoxyoctulosonic acid. The 2-keto-3-deoxyoctulosonic acid was determined by the thiobarbituric acid method (14). Protein concentrations were determined with a bicinchoninic acid protein reagent (Pierce Chemical Co., Rockford, Ill.; bulletin no. 25225) which tolerates detergents.

Treatment of whole GC, isolated OMs, and purified PI with proteases. Bacteria of strains 7122, 5766, and 1955 were suspended in phosphate-buffered saline (PBS) and adjusted to 200 Klett units. Samples of 300 \( \mu l \) of these cell suspensions were used for each test. Gonococci were centrifuged and resuspended in 100 \( \mu l \) of the same buffer. To each test sample 10 \( \mu l \) of TPCK-trypsin (660 \( \mu g/ml \)) in PBS and 0.3 mM CaCl\(_2\) were added and incubated for 30 min at 37°C. The cleavage of OMA of GC with alpha-chymotrypsin occurred with a protease concentration of 33 \( \mu g/ml \) for 30 min at 37°C in the presence of 0.3 mM CaCl\(_2\). Proteinase K dissolved in PBS with 0.3 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\) was used at a concentration of 8.25 \( \mu g/ml \). The proteinase K activity was stopped after 10 min at 37°C with 15 \( \mu g \) of phenylmethylsulfonyl fluoride. OMs were isolated as described above and adjusted to a membrane protein concentration of 0.5 mg/ml. The OMs were exposed to TPCK-trypsin, alpha-chymotrypsin, and proteinase K by the same methodology as described above for whole bacteria. The ratio of OM protein to protease was 7.5:1 for TPCK-trypsin, 15:1 for alpha-chymotrypsin, and 60:1 for proteinase K. Purified PI was dialyzed against glycine buffer as described above before reaction with proteolytic enzymes. The protein concentrations were adjusted to 0.75 mg/ml, and proteins were incubated with proteases at 37°C (as described above) for various times and at various enzyme concentrations. The ratio of PI to TPCK-trypsin was 20:1 (incubation time, 2 h), the ratio of PI to alpha-chymotrypsin was 10:1 (24 h), and the ratio of PI to proteinase K was 40:1 (30 min). The enzyme reactions had proceeded to completion after these incubation times except for that of proteinase K. These enzyme reaction time was chosen to obtain comparable cleavage fragments from the three protein molecules. A very short incubation time was chosen to maximize the total number of cleavage fragments observed. A prolonged proteinase K reaction of 1 h resulted in a total cleavage of PIA and PIB. All samples were prepared for SDS-PAGE, SDS gradient PAGE (10 to 20%), and peptide gels (8 M urea, 20% acrylamide, 0.1% SDS).

Western blot. The protein patterns were electrophoretically transferred from an SDS-polyacrylamide gel onto nitrocellulose paper as described by Towbin et al. (20). After blotting the nitrocellulose sheet was washed with PBS and blocked with 5% bovine serum albumin in PBS for 1 h at 37°C. MAbs were added and incubated for 1 h at 37°C. The nitrocellulose paper was washed again with PBS for 5 min and with PBS containing 0.05% Tween 20 and 0.5 M NaCl for 15 min. The nitrocellulose paper was rinsed further for 5 min with PBS and reacted with a secondary antibody (peroxidase-conjugated fraction of goat anti-mouse IgG) for 30 min at 37°C. This antibody solution contained 1% bovine serum albumin in PBS. After washing with PBS (5 min) and a PBS solution containing Tween 20 and NaCl as described above four times for 5 min at room temperature, color development was observed by adding 20 mM Tris hydrochloride buffer (pH 7.5) containing 0.06% (wt/vol) 4-chloro-1-naphthol and 0.06% (vol/vol) \( \text{H}_2\text{O}_2 \).

RESULTS

Purification of PI. The PI from three different strains was isolated as described above and further purified by gel filtration. The peak b fractions contained \( \leq 1\% \) LPS, as assessed by thiobarbituric acid assay. The PI peak contained also protein III and a few proteins with higher molecular weights (Fig. 1 and 2C, lane b).

Proteolytic treatment of whole GC, isolated OMs, and purified PI. PIA preparations of strain 7122 were exposed to TPCK-trypsin, alpha-chymotrypsin, and proteinase K. Intact GC were incubated with proteases for no longer than 30 min to prevent spontaneous cell lysis during the incubation time in PBS. OMs were treated with proteases in the same fashion as GC to obtain comparable results. Purified PI was exposed to proteases for different incubation times up to 24 h, so that the cleavage process could be monitored until no further enzyme digestion occurred. PIA's of whole GC and isolated OMs were not accessible to TPCK-trypsin and alpha-chymotrypsin (Fig. 2A and 2B), whereas proteinase K...
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alpha-chymotrypsin (C1, 32,000; glyceraldehyde-3-phosphate dehydrogenase GC unexposed purified PIA chymotrypsin; e, suspension cleavage exposed to bovine (45,000), exposed OM weight standards; b, pattern of isolated inhibitor purified PIA a, 100 by

FIG. 1. Elution profile obtained from a Sepharose 6B column. A 30-mg sample of OM protein was applied to the gel filtration column (2.6 by 100 cm) and eluted as described in the text. Peaks: a, membrane protein-LPS complex (contains maximal 25 µg of LPS per ml); b, PI and PII complex (maximal protein concentration, 0.8 mg/ml); c, protein, LPS, and DNA (maximal protein concentration, 40 µg/ml; maximal LPS concentration, 65 µg/ml; maximal DNA concentration, <0.1 µg/ml).

FIG. 2. Proteolytic cleavage of whole GC, isolated OM, and purified PIA of strain 7122. A, Whole GC of strain 7122 (300 µl of bacterial suspension with 200 Klett units) were exposed to proteases. Lanes: a, molecular weight standards alpha-lactalbumin (14,200), trypsin inhibitor (20,100), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), egg albumen (45,000), bovine albumine (66,000), and phosphorylase b (97,000); b, GC unexposed to proteases; c, GC exposed to TPCK-trypsin; d, GC exposed to alpha-chymotrypsin; e, GC exposed to proteinase K (P1, cleavage fragment of PIA; molecular weight 31,500). B, Cleavage pattern of isolated OM protein treated with proteases. Lanes: a, molecular weight standards; b, OM without enzyme treatment; c, OM exposed to TPCK-trypsin; d, OM exposed to alpha-chymotrypsin; e, OM exposed to proteinase K. C, Cleavage of purified PIA treated with proteases. Lanes: a, molecular weight standards; b, untreated PIA; c, PIA exposed to TPCK-trypsin (molecular weights: T1, 27,000; T2, 15,800); d, PIA exposed to alpha-chymotrypsin (C1, 32,000; C2, 27,200; C3, 25,500; C4, 15,000); e, PIA exposed to proteinase K (P1, 31,500; P2, 27,400; P3, 22,100; P4, 16,200; P5, 12,800).

split a fragment of approximately M, 1,000 off the protein of these preparations as confirmed by Barrera and Swanson (1). Purified PIA however was cleaved by all three enzymes, resulting in fragments of Mr 27,200 and 15,800 for TPCK-trypsin and 32,000, 27,200, 25,500, and 15,000 for alpha-chymotrypsin. These results are in contrast to earlier reports of Blake (2), where no cleavage was found with these enzymes. Treatment of purified PIA with proteinase K resulted in five fragments (31,500, 27,400, 27,100, 26,000, and 12,800) (Fig. 2C).

The PIB preparations of strain 5766 and 1955 treated with TPCK-trypsin, alpha-chymotrypsin, and proteinase K resulted in two to four cleavage fragments, whereas the purified PIB fractions were cleaved into three to five fragments (Table 1).

All PIB preparations were applied to peptide gels (8 M urea, 20% acrylamide, and 0.1% SDS) and revealed no additional fragments from those already seen in the 12.5% polyacrylamide–SDS gels. Time-dependent cleavage experiments with proteases, i.e., alpha-chymotrypsin, indicated that PIA was subsequently cleaved into the smallest of the observed fragments (Fig. 3).

Western blots. Most of the cleavage fragments of PIA (strain 7122) and PIB (strains 5766 and 1955) with molecular weights of ≥14,200 from whole GC, isolated OMs, and purified PIA were found to retain some reactivity (Fig. 4C, Table 1). Two to three protease K fragments of PIA and other cleavage fragments of PIB associated with intact bacteria or OM were unreactive or only lightly reactive with their respective MAb (Table 1, Fig. 4).

There was a general tendency for greatest antigenic reactivity to be manifested by the higher-molecular-weight fragments (Fig. 4).

DISCUSSION
PIA of intact GC and isolated OM and purified PIA are known to be resistant to proteolysis by TPCK-trypsin and alpha-chymotrypsin and less susceptible by treatment with proteinase K, whereas PIB is susceptible by proteolysis in
A revealed a good cleavage pattern of its weight (g) 25,500), PIA exposed to TPCK-trypsin (molecular weight: 27,200, C4, 15,000, C3, 23,300, C2, 18,800), and incubated for 90 min, (e) 2 h, (f) 24 h (C4, 32,000; C3, 25,500), (g) 31 h, or (h) for 48 h.

In contrast to the earlier report of Blake (2), our study revealed a good cleavage pattern of purified PIA after incubation with TPCK-trypsin (resulting in two fragments), alpha-chymotrypsin (four fragments), and proteinase K (five fragments). A possible explanation of the differences in our findings is that a different strain of gonococci was used in this study (7122) as compared with that used by Blake (120176-2).

There is little known about the correlation between susceptibility of PIA and the phenotypes of gonococcal strains. Different phenotypes of some strains might possess LPS molecules with different affinities for P1, possibly masking proteolytic cleavage sites. Also the low amount of LPS in the purified PIA preparation used in these studies might have facilitated access of the proteolytic enzymes to the PIA. The

**TABLE 1. Cleavage fragments of PIAs and PI Bs from different strains exposed to proteases**

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<tr>
<th>Strain</th>
<th>Prepn</th>
<th>No treatment</th>
<th>TPCK-trypsin</th>
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<sup>a</sup> Antigenic reactivity of cleavage fragments incubated with the monoclonal antibody reactive with that P1 serotype.

**FIG. 3.** Time-dependent cleavage of purified PIA (0.4 mg/ml) from strain 7122 with alpha-chymotrypsin (40 μg/ml). The molecular weight standards are shown in lane a. Other lanes: b, PIA not exposed to the enzyme and incubated for 48 h at 37°C; c through h, PIA exposed to protease for (c) 15 min (molecular weights: C2, 27,200; C3, 15,000), (d) 90 min, (e) 2 h, (f) 24 h (C4, 32,000; C3, 25,500), (g) 31 h, or (h) for 48 h.

**FIG. 4.** Immunostained PIA cleavage fragments with whole GC, isolated OMs, and purified PIA of strain 7122 exposed to proteases and reacted with MAb NG2D12A. A, Lanes: a, GC unexposed to proteases; b, GC exposed to TPCK-trypsin; c, GC exposed to alpha-chymotrypsin; d, GC exposed to proteinase K (PI, cleavage fragment of PIA, molecular weight, 31,500). B, Lanes: a, isolated OM unexposed to proteases; b, OM exposed to TPCK-trypsin; c, OM exposed to alpha-chymotrypsin; d, OM exposed to proteinase K (PI; molecular weight, 31,500). C, Lanes: a, purified PIA unexposed to proteases; b, PIA exposed to TPCK-trypsin (molecular weights: T1, 27,000; T2, 15,000); c, PIA exposed to alpha-chymotrypsin (C1, 32,000; C2, 27,200; C3, 25,500; C4, 15,000); d, PIA exposed to proteinase K (PI, 31,500; P2, 27,400; P3, 22,100; P4, 16,200).
results obtained after proteinase K treatment suggest for PIA that a segment of approximately only M, 1,000 or about eight amino acids, presumably located at one end of the molecule, is exposed on the surface of strain 7122, and that the remainder of the molecule is buried within the lipid bilayer of the OM. The lack of susceptibility of this exposed portion to proteolysis by TPCK-trypsin, alpha-chymotrypsin, or clostripain (Schmitt, unpublished data) indicates a lack of accessible lysine, arginine, or aromatic amino acid residues in this exposed region. The greater Western blot immunoreactivity of the M, 31,500 proteinase K fragment of PIA than of the native molecule with MAb NG2D12A suggests that this end fragment may participate in partially masking the epitope recognized by this MAb. The purified PIA molecules were also more slowly digested by each of the three proteases than either PIB molecule, as indicated by the higher-molecular-weight fragments isolated from PIA than PIB. This may reflect larger, predominantly hydrophobic regions in the PIA molecule, which would be consistent with a larger region buried within the lipid bilayer than for PIB and result in less availability of proteolytic target sites when the purified molecule was exposed to proteases in an aqueous environment. The fact that most of the PI (PIA and PIIB) fragments resulting from proteolytic enzyme cleavage retained the antigenic epitope recognized by the MAb (NG2D12A for strain 7122, NG23E9F for strain 5766, and G14B3F for strain 1955) suggests that these domains are relatively proteinase resistant.

These data indicate that PI (PIA and PIIB) is either cleaved from different sites, resulting in several fragments each carrying the specific epitope recognized by the MAb used in this study, or the domain is localized on multiple sites of the PIA and IB molecule. The MAbS used in our experiments are potent markers for the detection of surface exposed epitopes, which might prove to be candidates for the development of an effective vaccine against gonorrhea. We do not exclude the possibility that there may be other interesting PI epitopes exposed on the gonococcal cell surface which might be recognized by other MAbS.

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LITERATURE CITED