

ANTIGENIC AND IMMUNOGENIC PROPERTIES OF  
CYANOGEN BROMIDE PEPTIDES FROM GONOCOCCAL  
OUTER MEMBRANE PROTEIN IB

Evidence for the Existence of a Surface-exposed Conserved Epitope

BY TOM TEERLINK, HANNEKE VERSANTVOORT, AND E. COEN BEUVERY

*From the Departments of Bacterial Vaccines and Inactivated Viral Vaccines, National Institute of Public Health and Environmental Hygiene (RIVM), 3720 BA Bilthoven, The Netherlands*

Protein I is present in all strains of gonococci and is the most abundant outer membrane protein (1). Only a limited number of proteins I exist and the type of protein I expressed by a particular strain is a stable characteristic (2). Therefore, protein I is used to serotype gonococci (3, 4). The protein can be surface labeled with iodine (5, 6) or dansylchloride-cycloheptaamylose (7), demonstrating that it is partly exposed to the external environment. Antibodies against protein I have been shown to be bactericidal (8), and there is limited evidence that these antibodies may protect against salpingitis (9, 10). Recently, it has been shown (11, 12) that protein I can be translocated from the gonococcal outer membrane to the plasma membrane of eukaryotic cells. This phenomenon could possibly trigger endocytosis of gonococci by the host cell. Taken together these data make protein I a potential vaccine candidate.

Peptide mapping has shown that protein I contains conserved regions as well as unique sequences (5). These peptide maps could be divided into two groups. Experiments with proteolytic enzymes have confirmed the existence of two structurally different proteins I (13). Protein IA molecules have only one of their termini exposed to the environment, and show a limited susceptibility to proteolysis. Protein IB molecules have both termini buried in the membrane. The central region of protein IB is exposed and is susceptible to proteolysis by several enzymes (13–16). The same degradation pattern is observed, whether intact gonococci, isolated outer membranes, or purified proteins I are treated with these enzymes (14). On the basis of these observations a preliminary model for the structure of protein IB has been suggested (15, 16).

Only antibodies against epitopes located in the surface exposed part of protein I can be expected to contribute to protection against infection. Therefore, we have tried to define this region of protein IB more precisely and have studied its antigenic and immunogenic properties. We have fragmented protein IB by treatment with cyanogen bromide and have isolated the resulting peptides. The order of these peptides in the intact protein I was determined. Furthermore, experiments were performed to locate the position of the proteolytic cleavage sites relative to the cyanogen bromide cleavage sites. The reactivity of these

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peptides with antisera raised against purified protein I and against outer membrane complexes was determined.

The surface-exposed part of protein IB probably carries the serotype-specific determinants. It is not clear whether apart from these epitopes, common epitopes also reside in this region. To address this question we have raised antisera against surface-exposed peptides and studied the reactivity of these sera with heterologous outer membranes.

### Materials and Methods

*Gonococcal Strains and Growth Conditions.* The following gonococcal strains were used (serotypes within parenthesis): B2(1), 8658(2), 7929(3), 6611(4), C3(5), 8035(6), 5766N(7), F62(8), and F6(9). Bacteria were cultivated at 35°C in Frantz medium (17), supplemented with 0.2% yeast extract dialysate and 0.2% Casamino acids (Technical; Difco Laboratories Inc., Detroit, MI) in a 40-liter fermentor. The pH was maintained at 7.0, and pO<sub>2</sub> at 10%. Cultures were inactivated by heating at 56°C for 30 min. After centrifugation the bacteria were pooled and lyophilized. Outer membrane complexes (OMC)<sup>1</sup> were isolated from the cell-free culture supernatant as described before (18).

*Purification of Protein I.* Strain C3 was used to isolate protein I using the method described by Blake and Gotschlich (19).

*Cyanogen Bromide Cleavage.* Chemical cleavage of protein I at methionine residues with cyanogen bromide was performed by the method of Gross and Witkop (20). 10 mg of purified protein I was precipitated by addition of ethanol. The protein pellet was dissolved in 1 ml of 70% formic acid containing 100 mg of cyanogen bromide (Pierce Chemical Co., Rockford, IL). After incubation for 20 h at room temperature in the dark, the reaction mixture was dried under vacuum in a Speed Vac centrifuge (Savant Instruments, Hicksville, NY). The pellet was resuspended in water and dried again to remove traces of cyanogen bromide and formic acid.

*SDS-PAGE.* Samples were dissolved in 8 M urea and then diluted with an equal volume of sample buffer containing 0.125 M Tris-HCl (pH 6.8), 4% DTT, 20% glycerol, and 0.004% bromophenol blue. Samples were heated for 5 min at 100°C. Electrophoresis was performed on 2.5-mm gels by a modification of the method of Laemmli (21). The running gel contained 15% acrylamide, 0.75% bisacrylamide, and 4 M urea. The stacking gel contained 5% acrylamide and 0.13% bisacrylamide. A cyanogen bromide digest from myoglobin was used as molecular weight standard (Pharmacia Fine Chemicals, Uppsala, Sweden).

*Two-Dimensional Peptide Mapping.* A modification of the peptide mapping technique described by Pepinsky was used (22). 1 mg of protein I was partly degraded with cyanogen bromide (2 mg) for 30 min at room temperature. The reaction mixture was dried and dissolved in SDS-PAGE sample buffer. After electrophoresis, the gel was briefly stained with Coomassie brilliant blue and a longitudinal strip containing the separated fragments was excised. The entire gel strip was then incubated in a solution of 100 mg/ml cyanogen bromide in 0.1 M HCl. The cyanogen bromide was diluted from a stock solution of 600 mg/ml in 70% formic acid. After 1 h at room temperature the gel strip was washed four times for 15 min with 0.05 M Tris, pH 8.8, to remove unreacted cyanogen bromide. After a 10-min incubation in SDS-PAGE sample buffer at 60°C, the gel strip was placed on top of a second SDS-urea polyacrylamide gel, and electrophoresis was performed at right angles to the first dimension run. After electrophoresis the peptide spots were visualized by silver staining (23).

*Enzymatic Digestion.* Carboxypeptidase Y (Pierce Chemical Co.) was dissolved in 0.1 M sodium citrate buffer, pH 5.3. Tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin and chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) were dissolved in 1 mM HCl. Stock solutions were kept frozen before use. Proteolysis was performed by addition of enzyme to the purified protein I preparation (enzyme/substrate

<sup>1</sup> Abbreviation used in this paper: OMC, outer membrane complex.

ratio of 1:100): In the case of carboxypeptidase Y, the solution was diluted with an equal volume of 0.1 M sodium citrate buffer, pH 5.3. Incubation was carried out at 37°C for 2 h. Degraded protein was precipitated by additional of ethanol to a final concentration of 80%. The samples were then processed for SDS-PAGE or degraded with cyanogen bromide as described.

*Isolation of Peptides.* For preparative purposes gels were loaded with 150 µg/track of cyanogen bromide digestion mixture. After electrophoresis the gels were briefly stained with Coomassie brilliant blue to locate the peptide bands. These were excised, washed with water, and subsequently minced with a spatula. An equal volume of extraction buffer (8 M urea, 0.25 M Tris [pH 8.8], 1% (wt/vol) SDS) was added and the slurry was incubated for 10 min at 50°C. After centrifugation the supernatant was collected and the extraction procedure was repeated three more times. The combined supernatants were filtered through a 0.22-µm filter to remove residual gel particles. SDS and urea were removed by precipitating the peptides with 90% acetone. Purity of peptides was checked by analytical SDS-PAGE.

*Preparation of Antisera.* Groups of eight random-bred mice (strain Cpb:SE) were immunized intraperitoneally. Protein I was denatured by addition of excess SDS and boiling for 5 min. OMC, native protein I, and denatured protein I were adsorbed to a 100-fold excess of AlPO<sub>4</sub> and used in a 1-µg dose. Cyanogen bromide peptides were suspended in 0.15 M NaCl, emulsified with an equal volume of CFA, and injected in a 10-µg dose. Booster injections were administered after 6 wk using the same dose and route. 2 wk later the mice were bled. Sera were pooled and stored at -20°C.

*Absorption of Antisera.* To free antisera from antibodies capable of reacting with the native outer membrane, sera were absorbed with OMC. The sera were diluted 1:100 with 0.9% NaCl and incubated with OMC at several concentrations (see Results). After 1 h at 37°C and 16 h at 4°C the OMC were removed by centrifugation (1 h at 300,000 g). The supernatants were tested by ELISA and immunoblotting.

*Enzyme-linked Immunosorbent Assay (ELISA).* Polyvinylchloride microtiter plates were coated overnight at room temperature. OMC were coated at a concentration of 5 µg/ml in PBS. Purified peptides were dissolved in a small volume of 8 M urea and subsequently diluted in 50 mM sodium carbonate buffer (pH 9.6) to a protein concentration of 0.5–1.0 µg/ml. The final urea concentration was <10 mM. After washing with PBS containing 0.02% Tween-80, remaining adsorption sites were blocked by a 1-h incubation with 0.5% BSA in PBS. The plates were subsequently incubated for 1 h each with serum and goat anti-mouse IgG conjugated to horseradish peroxidase. The sera were diluted with washing buffer containing 0.5% BSA. 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) was used as substrate. OD<sub>450</sub> was determined on an Titertek Multiscan immunoassay reader (Flow Laboratories, Inc., McLean, VA).

*Immunoblotting.* Cyanogen bromide-digested protein I was subjected to SDS-PAGE (1 µg of protein per track). The peptides were transferred to nitrocellulose using the Western blotting technique of Burnette (24) with the buffer system described by Towbin (25). Electrophoretic transfer was performed for 2 h at 500 mA. After transfer, the nitrocellulose was cut into strips that were washed overnight in PBS with 0.5% Tween-80 (washing buffer). The strips were then incubated for 60 min with mouse antisera diluted in washing buffer containing 0.5% (wt/vol) BSA. Usually a 1:500 dilution was used. After washing for 60 min the strips were incubated with peroxidase-conjugated goat anti-mouse IgG (diluted 1:1,000 in the BSA-containing washing buffer). The nitrocellulose strips were then washed again for 60 min. The strips were developed with a diocylsulfosuccinate-tetramethylbenzidine substrate (26).

*Analytical Methods.* Protein was determined by a modification of the method of Lowry et al. (27) as described by Peterson (28). Alternatively, a commercial protein assay kit (Pierce Chemical Company) was used (29). BSA was used as standard.

## Results

*Ordering of the Cyanogen Bromide Fragments of Protein I.* When protein IB (strain C3, serotype 5) was treated with cyanogen bromide five fragments were

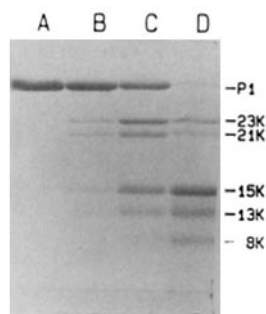


FIGURE 1. SDS-PAGE analysis of cyanogen bromide-degraded protein I. Protein I was degraded with a 10-fold excess of cyanogen bromide. Samples were taken after 0 min (A), 10 min (B), 30 min (C), and 24 h (D).

produced. These fragments were resolved by SDS-urea polyacrylamide gel electrophoresis as shown in Fig. 1. The gel system used allows the separation of peptides with a molecular weight down to  $\sim 2,000$ . Therefore, all major fragments are detected in this way. When the cyanogen bromide concentration or reaction time were increased the intensity of the upper two fragments diminished, with a concomitant increase in intensity of the three smaller peptides, but no additional fragments were generated. The three lower peptides, having  $M_r$  of  $\sim 8,000$ ,  $\sim 13,000$ , and  $\sim 15,000$ , are therefore final cleavage products. The upper two fragments result from incomplete cleavage of protein I. We have used the incomplete cleavage products to deduce the order of the peptides in the intact protein I. To this end, protein I was treated with a limited amount of cyanogen bromide to assure the presence of a substantial amount of incomplete cleavage products in the reaction mixture. After separation of the peptides by SDS-PAGE as in Fig. 1, an entire longitudinal gel strip was excised and treated with a high concentration of cyanogen bromide to further cleave the intermediary products. The entire gel strip was then placed on top of a second SDS-urea slab gel and electrophoresis was performed at right angles to the first dimension run. Fig. 2 shows the resulting pattern of peptide spots. Complete cleavage products resist cleavage in the second cyanogen bromide incubation and therefore yield a single spot on the diagonal. Partial cleavage products are degraded further and yield more spots. The three peptides of low molecular weight were found to be resistant to the second cleavage and therefore are final cleavage products. One of the incomplete cleavage products was split into fragments of 8,000 and 13,000 mol wt, whereas the other one was split into fragments of 8,000 and 15,000 mol wt. From these results we conclude that the 8,000 mol wt fragment is flanked by both the 13,000 and the 15,000 mol wt fragments and is consequently in the middle of the intact protein I molecule. Although the three final products account for the total molecular weight of protein I, it cannot be excluded that an additional small peptide ( $< 2,000$  mol wt) is generated that is not detected in our SDS-PAGE system. However, if this were the case we would expect to see additional intermediary products appearing as doublets on the gel. As no doublets were visible this implies that additional peptides are either very small (i.e., smaller than five amino acids) or absent.

To determine which of the larger peptides is derived from the  $\text{NH}_2$ -terminus, protein I was first treated with carboxypeptidase Y and subsequently with cyanogen bromide. The SDS-PAGE profile of the resulting peptides revealed

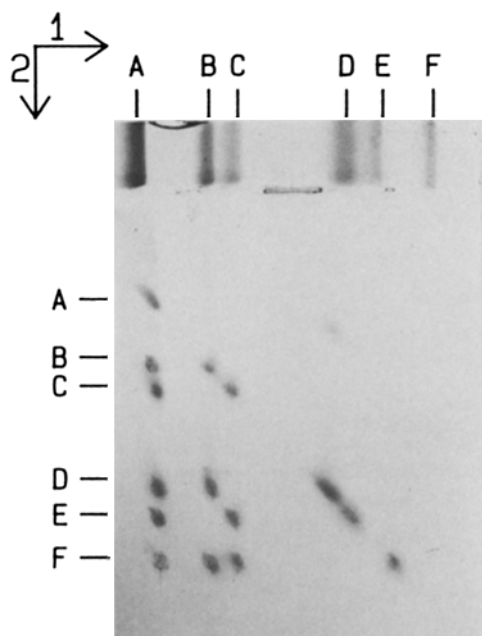


FIGURE 2. Ordering of cyanogen bromide peptides from protein I by two-dimensional electrophoretic analysis. Protein I was degraded with a twofold excess of cyanogen bromide for 30 min and the resulting peptides were separated by SDS-PAGE and silver stained. A duplicate gel strip was treated with 100 mg/ml cyanogen bromide and the resulting fragments were electrophoresed into an SDS-urea slab gel. Peptide spots were visualized by silver staining. (A) Undegraded protein I; (B and C) intermediate cleavage products; and (D-F) complete cleavage products.

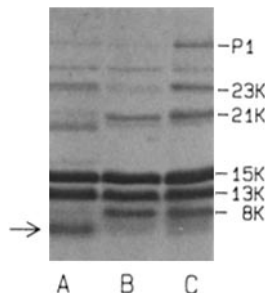


FIGURE 3. SDS-PAGE analysis of a combined proteolytic and chemical cleavage of protein I. The following combinations were used: (A) trypsin followed by cyanogen bromide; (B) chymotrypsin followed by cyanogen bromide; (C) cyanogen bromide. Native protein I was treated with the indicated enzyme. After precipitation the product was degraded with cyanogen bromide.

that the 15,000 mol wt peptide was shifted to a lower molecular weight. Therefore, this peptide forms the COOH-terminal part of protein I and the 13,000 mol wt peptide is derived from the NH<sub>2</sub>-terminus.

*Location of the Sites Susceptible to Proteolysis.* It has been observed (15) that when outer membranes are treated with trypsin or chymotrypsin, protein I is cleaved at a limited number of sites, yielding two products that remain associated with the membrane. The same cleavage pattern is observed when purified protein I is treated with these enzymes. Apparently the detergent micelles take over the role of the outer membrane phospholipids in shielding large parts of the protein and protecting it against proteolysis. We have determined the cyanogen bromide peptides that contain these cleavage sites by a combination of enzymatic and chemical degradation of protein I. Protein I was first treated with either trypsin or chymotrypsin. Then the product was further degraded by treatment with cyanogen bromide. Fig. 3 shows the resulting fragmentation patterns. When cyanogen bromide treatment was preceded by an incubation with trypsin, both 15,000 and 13,000 mol wt fragments remained unaltered, but the 8,000 mol wt

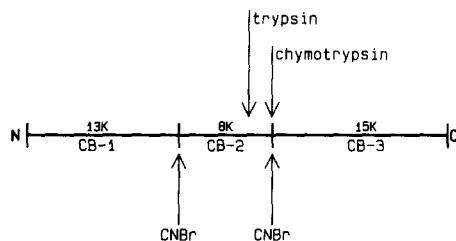


FIGURE 4. Preliminary peptide map of protein IB. The cleavage sites for trypsin, chymotrypsin, and cyanogen bromide are indicated with arrows. The peptides obtained after cyanogen bromide degradation are numbered CB1 to CB3, starting from the NH<sub>2</sub>-terminus.

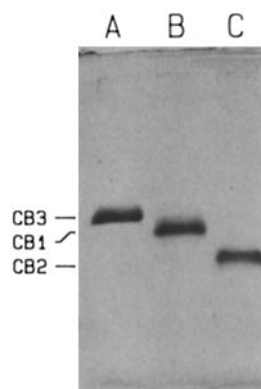


FIGURE 5. SDS-PAGE analysis of purified cyanogen bromide peptides from protein I. Protein I was degraded with a 10-fold excess of cyanogen bromide for 24 h and the resulting peptides were separated on a preparative SDS-urea slab gel. Peptide bands were excised and extracted as described in Materials and Methods. Purity of the peptides was assessed by analytical SDS-PAGE. (A) CB3, (B) CB1, and (C) CB2.

fragment was replaced by a peptide having a molecular weight of ~6,000. Apparently, the 8,000 mol wt fragment contains the tryptic cleavage site. When chymotrypsin was used instead of trypsin, all three peptides remained unaltered. Therefore, the chymotryptic cleavage site is located at or very near to one of the cyanogen bromide cleavage sites. From the fact that the largest intermediary product of 23,000 mol wt was very much reduced in intensity, it can be concluded that this site is located between the 15,000 and the 8,000 mol wt fragment.

Fig. 4 schematically summarizes the information obtained from these experiments. The cyanogen bromide peptides are numbered CB1, CB2, and CB3, starting from the NH<sub>2</sub>-terminus. The proteolytic cleavage sites are located in CB2 and close to the junction between CB2 and CB3. We therefore assume that this region is part of the hydrophilic loop of protein I that protrudes from the membrane.

*Purification of Peptides.* To study the antigenic properties of the cyanogen bromide peptides by ELISA, we have attempted to purify the individual peptides. A major problem during the purification was the very hydrophobic nature of both CB1 and CB3, causing them to coelute in various chromatographic systems. Reverse-phase chromatography in the presence of 60% formic acid resulted in the isolation of CB2 in pure form, but both other peptides were eluted together. In view of these problems we decided to isolate the peptides by preparative SDS-PAGE. The peptides were extracted from the gels with a buffer containing 8 M urea and 1% SDS (see Fig. 5).

*Antigenic Activity of the Cyanogen Bromide Fragments.* After removal of urea and SDS by acetone precipitation, the peptides were used to coat microtiter plates for ELISA. Fig. 6 shows the reaction of a number of antisera with the three peptides. It can be seen that antiserum raised against purified protein I reacted

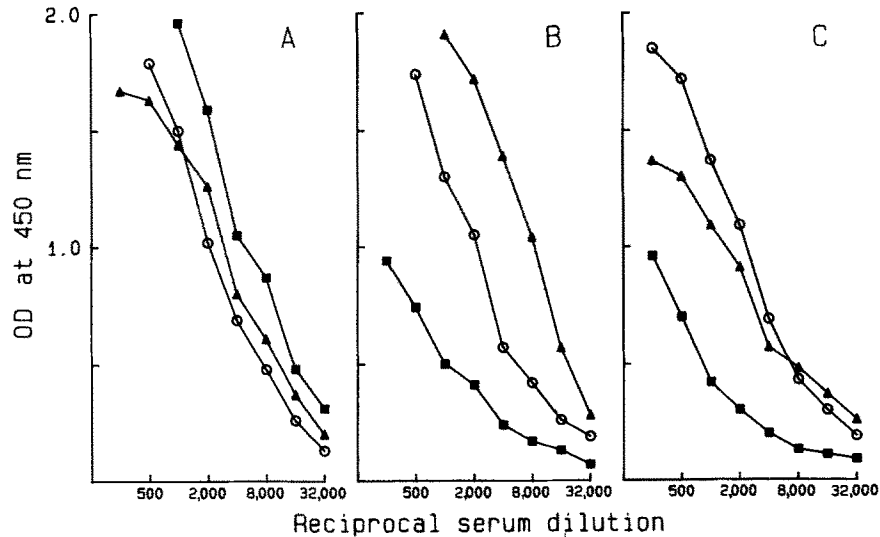


FIGURE 6. ELISA titration curves of several sera. Microtiter plates were coated with CB1 (A), CB2 (B), and CB3 (C). The following sera were tested: anti-OMC (triangles), anti-native PI (circles), and anti-SDS-denatured PI (squares).

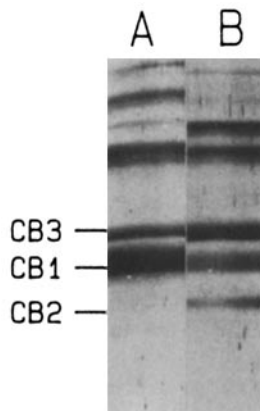


FIGURE 7. Western blot analysis of sera raised against SDS-denatured protein I (A) and native protein I (B). Note the strong reaction with CB1 and the very weak reaction with CB2 in A as compared with B.

well with all three peptides, whereas anti-SDS-denatured protein I reacted strongly with CB1 but only moderately with the other peptides. Antisera against the outer membrane complex reacted with all three peptides, showing a relatively strong reaction with CB2. Probably this region of protein I is very immunogenic when present in the outer membrane.

We have also used the immunoblotting technique to detect the presence of antibodies against the three peptides in the aforementioned antisera. The results are shown in Fig. 7. Antiserum against purified protein I reacted with all three cyanogen bromide peptides. On the other hand, an antiserum raised against SDS-denatured protein I reacted very strongly with CB1 and displayed only a moderate reaction with both other peptides. Apparently CB1 contains an epitope that becomes immunodominant once the protein is denatured with SDS. These results clearly confirm the ELISA results.

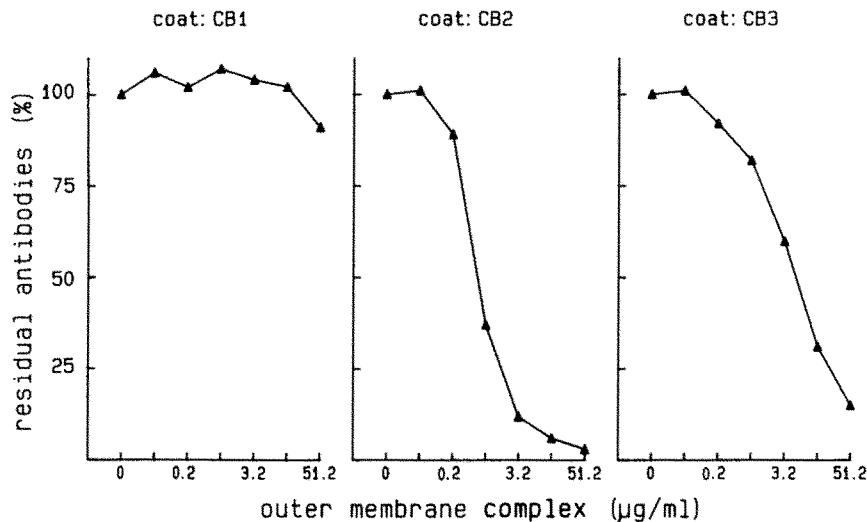


FIGURE 8. Absorption of antibodies against the cyanogen bromide peptides from protein I by native outer membranes. Antiserum against native protein I was incubated with various amounts of OMC. After incubation, residual antibodies against CB1 (*left*), CB2 (*middle*), and CB3 (*right*), were determined by ELISA.

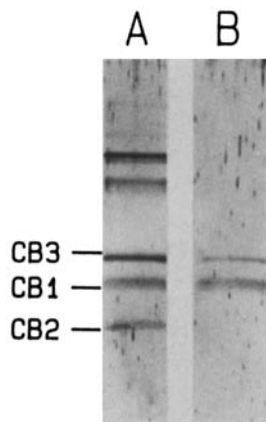


FIGURE 9. Western blot analysis of antiserum raised against native protein I before (*A*) and after (*B*) absorption with homologous OMCs. Note that antibodies against CB3 are partly absorbed and antibodies against CB2 are completely absorbed.

These experiments demonstrated the presence of antibodies against individual peptides in sera raised against purified protein I. To determine whether these antibodies could also react with protein I present in the outer membrane, absorption experiments were performed. Antiserum raised against protein I was first absorbed with homologous OMC and then the residual antibodies against the three peptides were determined by ELISA (Fig. 8). Antibodies against CB2 were completely absorbed by the OMC, in accordance with its surface-exposed orientation. Antibodies against CB3 were also absorbed, although much higher amounts of OMC were required. Anti-CB1 antibodies were not absorbed at all, suggesting that this peptide is not exposed at the membrane surface. The absorbed antiserum was also analyzed by immunoblotting. Fig. 9 shows that when the serum against purified protein I was absorbed, the antibodies reacting with CB2 were completely removed. Reaction with CB3 was very much reduced,



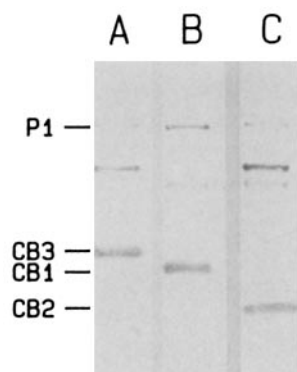


FIGURE 10. Western blot analysis of antisera raised against purified cyanogen bromide peptides from protein I. (A) anti-CB3; (B) anti-CB1; (C) anti-CB2.

TABLE I  
Ability of Antiserum Raised against CB2 (Serotype 5) to React with Heterologous Outer Membranes as Determined by ELISA

Serotype OMC	ELISA reading*
1	0.26
2	0.18
3	0.20
4	0.92
5	1.05
6	1.75
7	0.50
8	0.12
9	0.06

\* OD<sub>450</sub> at a serum dilution of 1:400.

but the reaction with CB1 was hardly diminished. These results show that when purified protein I is used to generate an antiserum, antibodies against epitopes residing in CB2 and CB3 are induced that can also react with the native outer membrane. Antibodies against CB1 do not react with the outer membrane. Probably this fragment of protein I is buried in the membrane and is therefore not available for reaction with antibodies.

We have also determined the presence of antibodies against the cyanogen bromide peptides in antisera that were raised against OMCs of heterologous gonococcal strains. These sera contained only very low amounts of antibodies reacting with the serotype 5 cyanogen bromide peptides. Apparently, mainly serotype-specific antibodies are induced after immunization with outer membranes.

*Immunogenic Activity of CB2.* Antisera raised against heterologous OMC contained only very low amounts of antibodies against the serotype 5 CB2 peptide. This could mean that the surface-exposed part of protein IB contains only serotype-specific epitopes. Another possibility is that common epitopes do exist but are not immunogenic when OMC are used as immunogen. To investigate these possibilities we raised an antiserum against the isolated CB2 fragment and tried to determine the presence of crossreactive antibodies in this serum. Fig. 10 shows an immunoblotting experiment using sera raised against the three cyanogen bromide peptides. It can be seen that the sera react only with the peptides

used for immunization and with the intermediary products containing these peptides. We were able to show that the serum against CB2 reacted with the homologous native outer membrane. Table I shows the reaction of this serum with heterologous OMC as determined by ELISA. It can be seen that the serum reacted strongly with OMC of serotypes 4, 5, 6, and 7. Therefore, proteins IB of these serotypes share at least one epitope that is surface exposed.

### Discussion

It has been shown (15) before that when intact gonococci or isolated outer membranes are treated with proteolytic enzymes, protein I is cleaved into two fragments that remain associated with the membrane. On the basis of this observation Blake et al. (15, 16) have suggested that protein I has a looplike structure with both termini buried in the membrane and the central part of the protein exposed to the environment. This model was suggested for protein IB, present in strains of the WII/WIII serogroup. Protein IA, expressed by strains of the WI serogroup has been shown (13) to have one of its termini exposed on the surface of the membrane. The aim of this study was to define the surface-exposed part of protein IB in more detail and to determine whether this region contains conserved epitopes. All experiments were performed with a serotype 5 strain.

We have found that after degradation with cyanogen bromide three fragments are formed, having  $M_r$  of 8,000, 13,000 and 15,000. Using a special peptide-mapping technique we have determined the order of these peptides in the intact protein I (see Fig. 2). A combination of proteolytic and chemical degradation of protein I revealed that the middle peptide (8,000  $M_r$ ) contains the site where trypsin splits the native protein. The cleavage site for chymotrypsin was found to be located at the junction between the 15,000 and the 8,000 mol wt fragment (see Fig. 4). We therefore propose a model for protein I in which the  $\text{NH}_2$ -terminal part of the 15,000 mol wt fragment and a large part of the 8,000 mol wt fragment are part of the surface-exposed region.

Antisera were raised against OMCs, purified protein I, and SDS-denatured protein I. These sera were tested for reactivity with the three cyanogen bromide peptides by immunoblotting and ELISA. Some interesting quantitative differences between these sera were observed. Antiserum against the denatured protein I reacted very strongly with CB1, whereas the other two sera showed only a moderate reaction with this peptide. Apparently this peptide is not very immunogenic in the native protein, probably because it is not accessible on the surface. It may be that it is buried in the tertiary structure of protein I, or that it is located at the interface between the protein I molecules in the native trimer. Once the protein is denatured this region becomes immunodominant. It was also shown that antibodies against CB1 cannot react with the native outer membrane (Fig. 8). This observation is in agreement with CB1 being buried in the membrane or in the protein structure. Antibodies against CB2 and CB3 were shown to be bound by the native outer membrane, confirming their surface-exposed nature. The fact that relatively large amounts of antibodies against CB2 are induced by immunization with OMC also suggests that this peptide is surface exposed.

After immunization with OMC, mainly serotype-specific antibodies are

formed. Sera raised against heterologous OMC hardly react with the serotype 5 CB2 peptide. Therefore, the surface-exposed part of protein IB contains only type-specific epitopes, or alternatively, common epitopes are present but are immunorecessive. We were able to demonstrate the existence of common epitopes by immunization with CB2. The serum reacted well with several OMC within the WII/WIII serogroup (i.e., serotypes 4–7).

Comparison of the immunochemistry of protein I with that of the gonococcal pilus reveals some interesting similarities. The amino acid sequence of the pilus has been determined (30) and its immunochemistry has been studied in detail (31–34). Pili, like protein I, contain both conserved and variable antigenic determinants. The pilin subunit contains an immunodominant variable region and a common domain that is hardly immunogenic. Studies with a panel of mAbs showed that crossreactive antibodies bound to SDS-unfolded pilin, whereas type-specific antibodies bound significantly less. Therefore, the crossreactive antibodies recognize the conserved primary structure, whereas the type-specific antibodies probably recognize part of the tertiary structure of the variable region (32). It may be that the antigenic determinants of protein I also fall into two classes: On the one hand, serotype-specific determinants that are conformation dependent, and on the other hand, common epitopes that are linear. Antibodies against the conformation-dependent epitopes are induced by immunization with native protein I or outer membranes. Antibodies against the common epitopes are more readily induced by immunization with denatured forms of protein I or isolated peptides. That the variable surface-exposed part of protein I contains a conserved epitope is not without precedent. Within the variable domain of the pilin subunit a common epitope was detected with an mAb (33).

Recently, another group has succeeded in raising an mAb against a surface-exposed common epitope of protein IB (35). This mAb was shown to be bactericidal and opsonic (36). The epitope was destroyed by treatment of protein I with chymotrypsin but not by treatment with trypsin. Although the authors used another strain (P9), the proteolytic cleavage pattern very much resembles that of strain C3 used in our study. This would mean that the epitope is located close to the junction between CB2 and CB3. Currently, we are raising mAbs against the individual cyanogen bromide peptides. The availability of a panel of these mAbs will aid in locating the conserved epitope with more precision.

### Summary

Two distinct species of gonococcal porin proteins exist that differ with regard to surface exposure. Protein IB, expressed by strains of the WII/III serogroup, has both termini buried in the outer membrane, leaving a central region of the molecule exposed at the cell surface. We have attempted to define this region of protein IB in detail by studying the antigenic and immunogenic properties of peptides derived from protein IB. Treatment of gonococcal protein IB (serotype 5) with cyanogen bromide resulted in cleavage of protein IB into three major fragments of  $M_r$  of 15,000, 13,000, and 8,000. The location of these peptides in the intact protein was determined by analysis of partial cleavage products. The 8,000  $M_r$  peptide (CB2) was found to be located in the central region of the protein. Chymotrypsin cleavage of protein IB revealed a cleavage site near one

of the cyanogen bromide cleavage sites. Trypsin was found to cleave the protein, either in outer membranes complexes (OMC) or in detergent micelles, in the central CB2 fragment. These results suggest that CB2 is a part of the surface-exposed region of protein IB.

Immunization of mice with purified protein IB (serotype 5) induced antibodies against all three CB-peptides. Absorption of the sera with homologous OMC resulted in a complete removal of antibodies against CB2, supplying further evidence for its surface-exposed nature. Antibodies against the 13,000  $M_r$  peptide (CB1) could not be absorbed with intact OMC, suggesting that this peptide is buried within the outer membrane.

Antisera raised against CB2 of serotype 5 demonstrated a considerable cross-reactivity with heterologous outer membranes. On the contrary, intact OMC induced mainly type-specific antibodies. These data demonstrate the presence of conserved epitopes on the surface-exposed CB2 peptide. These conserved epitopes are generally not very immunogenic when present in intact OMC.

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