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ANTIGENIC SUBUNIT OF THE POLYPEPTIDE ANTIGENIC COMPLEX OF THE MELVIN STRAIN OF NEISSERIA GONORRHOEAE

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SUMMARY

An antigenic subunit of molecular weight 66,000 daltons has been isolated from the antigenic complex of the Melvin strain of Neisseria gonorrhoeae. Incubation of the complex in 8M urea at room temperature for four hours resulted in the dissociation of the subunit from the complex. It was separated from the complex by chromatography of the incubation mixture on a Sepharose 6B column in 50 mM ammonium bicarbonate pH 8.5 without 8M urea and further purified by affinity chromatography. This communication reports on a newly isolated antigenic protein devoid of LPS present in the bacteria.

The cell envelope of gram-negative bacteria consists of three morphologically distinct layers, the outer L layer, the middle peptidoglycan layer and inner cytoplasmic layer. Since several antigenic components are either attached to or present in the outer layer, this layer has become the subject of many investigations (1-11). Because of their antigenicity and presumed role in the pathogenicity of the disease, outer membrane proteins are considered attractive vaccine candidates. In our study of the structure and functional relationship of these proteins, we recently reported isolation of the antigenic complex from the Melvin strain of Neisseria gonorrhoeae (7). The complex is a polypeptide composed of several subunits of molecular weight 110,000 to 12,000 daltons, some of which are outer membrane proteins. In the present communication we report isolation of an antigenic subunit with a molecular weight of 66,000 daltons which is devoid of LPS. Evidence is also presented that during the isolation of the complex some of the subunit is obtained in a monomeric form separated from the parent complex.

Abbreviations used are: SDS, sodium dodecyl sulfate; KDO, 2-keto-3-deoxyoctonic acid; LPS, lipopolysaccharide; BSA, bovine serum albumin; antigenic complex, complex.

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MATERIALS AND METHODS

The Melvin strain of Neisseria gonorrhoeae was obtained from the Center for Disease Control in Atlanta, Georgia. The cells were grown in a medium according to Frantz (12) and were harvested at the stationary phase. Agarose A-1.5M and Sepharose 6B were purchased from Bio-Rad Laboratories and Pharmacia Fine Chemicals, respectively; Dulbecco's phosphate buffered saline (1x) was from Grand Island Biological Company.

For isolation of the complex the cell paste was mixed with seven volumes of 200 mM Tris-HCl buffer pH 7.5 and was homogenized in a Sorvall Omni-Mixer for one minute at setting 1. During this operation, the container was kept under ice; the cells were extracted at 4°C for 2 hours. The suspension was centrifuged at 39,100 xg for 30 minutes and the pellet was discarded. The supernatant was chromatographed on an Agarose A-1.5M column at room temperature equilibrated with 15 mM NaCl. The fractions at the void volume containing the complex were pooled and chromatographed on a Sepharose 6B column equilibrated with the same eluent. The latter chromatography facilitated the separation of the complex from trace amounts of second peak material.

Antisera to the gonococcus cell were prepared in New Zealand white rabbits. A suspension of 1 x 10^9 live cells of the bacteria was made in 0.2 ml, 0.5 ml, 1 ml and again 1 ml of 15 mM NaCl and injected intravenously on day 1, 3, 10 and 14, respectively. A test bleed was taken one week following the day 14 injection. Sera showing acceptable levels of antibody were used for the immunodiffusion assay.

Antisera to the subunit were prepared in New Zealand white rabbits. Initial intramuscular inoculations consisted of 1 mg of the second peak material (Fig. 3, Fractions 42-60) in Dulbecco's phosphate buffered saline (1x), pH 7.0 emulsified in an equal volume of complete Freund's adjuvant. Fourteen days later the animals received a booster dose of the same concentration of immunogen emulsified in an equal volume of incomplete Freund's adjuvant. Sera with good antibody activity were obtained following the second injection. The animals were bled approximately every seven days.

All sera were stored at -20°C. Ouchterlony double-diffusion reactions were performed using Hyland Immuno-Plate, Immunodiffusion Plate Pattern D. Antibody produced in rabbits against the subunit fraction was partially purified by ammonium sulfate precipitation (13) and was coupled to CNBr-activated Sepharose 4B following the instructions in the Pharmacia technical bulletin. The antibody-coupled gel was poured into a column and was equilibrated with 10 mM ammonium bicarbonate, pH 7.4. The lyophilized second peak material from the Sepharose 6B column chromatography (Fig. 3, Fractions 42-60) was dissolved in ammonium bicarbonate buffer and was applied to the column. After the optical density of the breakthrough peak reached zero, the bound subunit was eluted with 5M guanidine hydrochloride.

SDS-polyacrylamide gel electrophoresis was done according to Laemmli using a 12% gel (14). Gels were stained with 0.25% Coomassie brilliant blue and destained as described by Weber and Osborne (15). For KDO determination a procedure of Karkhanis et al. (16) was followed. Protein was determined by the Lowry method (17) using bovine serum albumin as the standard. The amino terminal analysis was done by treatment of the protein with Dansyl chloride (18).

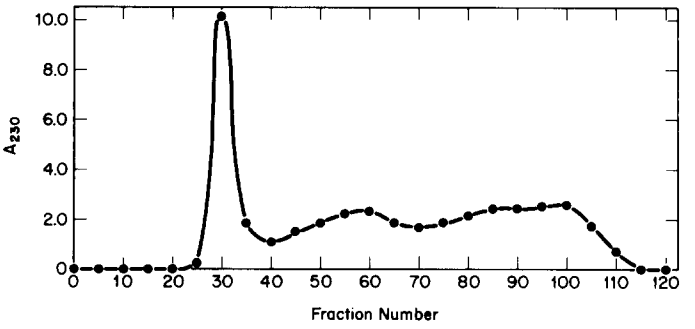

Figure 1. Chromatography of the crude extract obtained from gonococcus on A-1.5M column (8 x 30 cm). Seventy-five ml of the extract containing 500 mg protein were applied to the column. The flow rate was 30 ml/hr. Each fraction was 13 ml in volume. The chromatography was carried out at room temperature in 50 mM ammonium bicarbonate, pH 8.5.

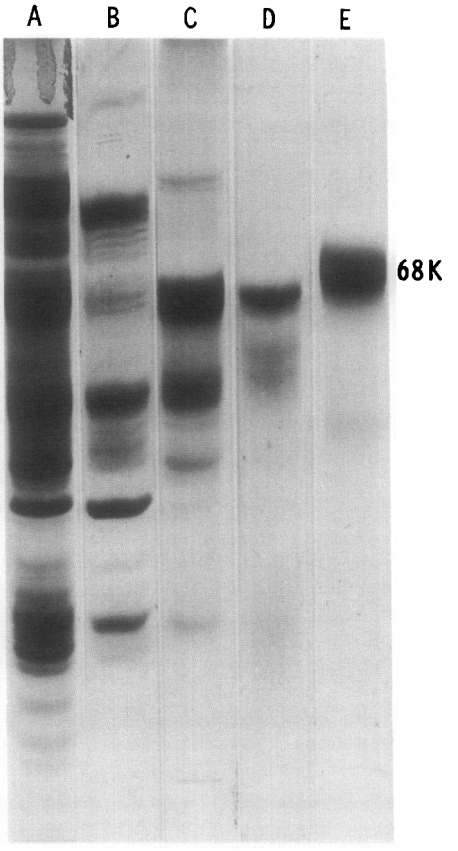

Figure 2. SDS-polyacrylamide electrophoresis of fractions from A-1.5M, Sepharose 6B and affinity chromatographies. A, parent complex (200 µg); B, first peak material, Fig. 3, fractions 20-32 (200 µg); C, second peak material, Fig. 3, fractions 42-60 (200 µg); D, purified subunit, Fig. 4, fractions 14-20 (100 µg); E, BSA (15 µg).

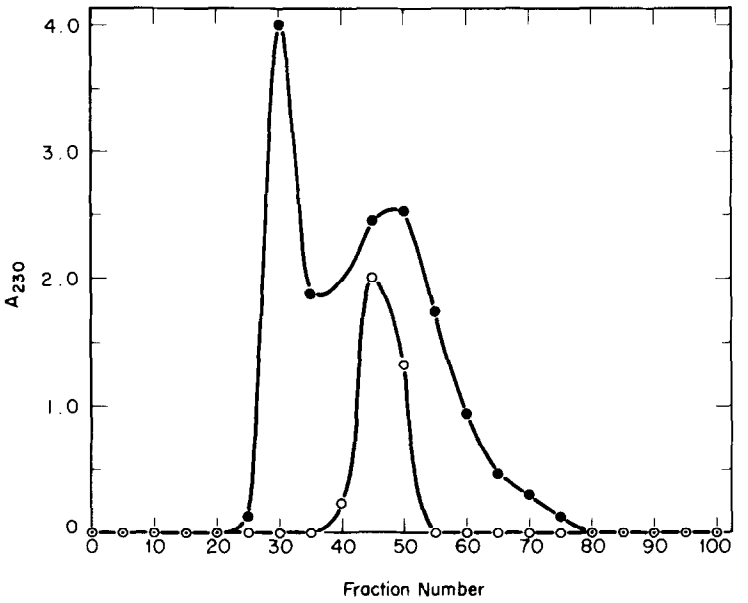

Figure 3. Chromatography of the parent complex after treatment with 8M urea. Thirty-three mg of the complex were incubated in 3.3 ml of 50 mM ammonium bicarbonate, pH 8.5 for four hours at room temperature and applied to a Sepharose 6B column (2.5 x 41 cm). The flow rate was 58 ml/hr. Each tube contained a fraction of 2 ml in volume. The chromatography was carried out at room temperature. ●-●, parent antigen; 0-0, BSA 10 mg/ml.

RESULTS

Fig. 1 shows the chromatography of the Tris-HCl extract of the bacteria on an A-1.5M column. Three peaks were obtained. The first peak (Fractions 27-35) contained the parent antigenic complex which was further purified on a Sepharose 6B column (data not shown). The SDS gel electrophoresis of the complex shows that it consists of several subunits (Fig. 2A). The lyophilized preparation of the complex was incubated in 8M urea at room temperature for four hours which resulted in the dissociation of the subunit from the complex (Fig. 3); this subunit was further separated by Sepharose 6B chromatography. The first peak contained most of the subunits (Fig. 2B), while the second peak contained predominantly a subunit of 66,000 daltons (Fig. 2C). To further purify the second peak material, it was chromatographed on an affinity column (Fig. 4). The subunit which was bound to the gel was eluted with 5M

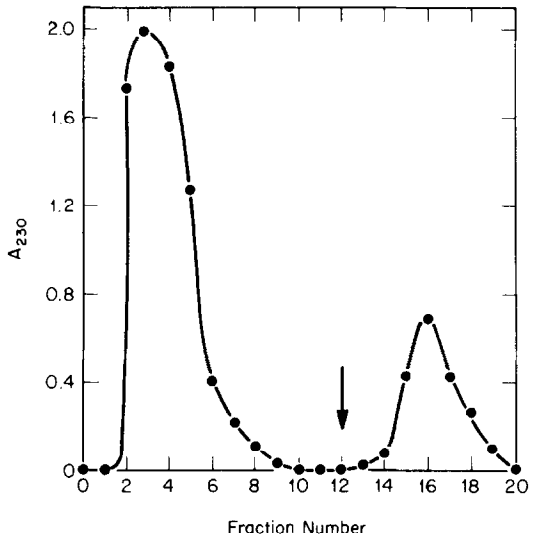

Figure 4. Affinity chromatography of the subunit fraction (Fig. 3, second peak). Six mg dissolved in 2 ml of 10 mM ammonium bicarbonate, pH 7.4 were applied to the column (1.1 x 2.2 cm) equilibrated with the same buffer. At the arrow shown the column was eluted with 5M guanidine hydrochloride to obtain the subunit.

guanidine hydrochloride. This gave a highly purified subunit (Fig. 2D), which has a molecular weight of 66,000 as compared with BSA (Fig. 2E). The observation of the presence of a single amino terminal, aspartic acid, obtained by dansylation is consistent with this data.

When the fractions were tested in the Ouchterlony immunodiffusion assay using antibody to the whole cell, the parent complex gave two lines, one near the antigen well and the other between the antigen and antibody well (Fig. 5A). The subunit showed identity with the second line between the antigen and antibody (Fig. 5B). It was also identical to the material present in the second peak (Fractions 40-60) of the A-1.5M chromatography (Fig. 5C). When an immunodiffusion assay was carried out against antibody to the subunit, such identity was evident (Fig. 5E, F, G). Table 1 demonstrates that no KDO was present in the subunit while there was three-fold increase in the KDO content of the remaining complex after removal of the subunit. The assay used for KDO determination had a lower limit of detection of 0.06% (16). The KDO content is a reflection of the amount of LPS present in these components.

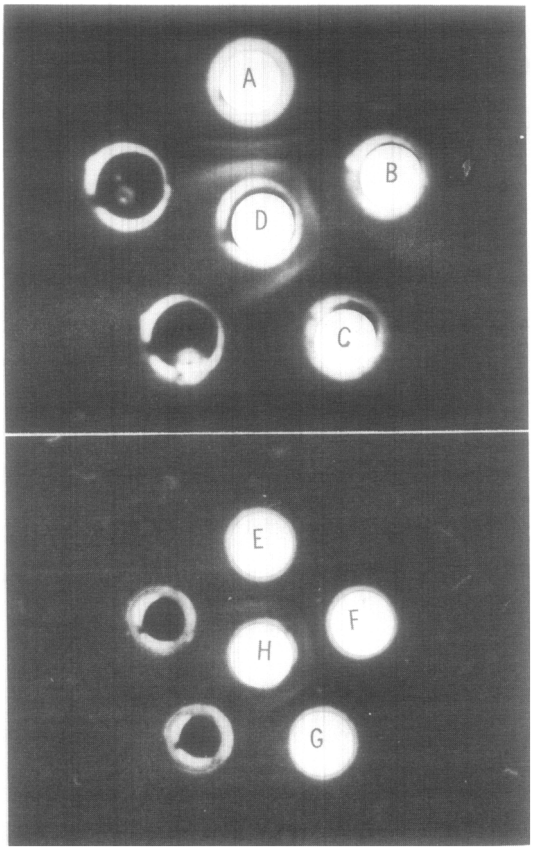

Figure 5. Ouchterlony immunodiffusion of different antigenic fractions. Well A, parent complex; B, purified subunit; C, fractions 40-60, Fig. 1; D, antisera to the whole cell; E, parent complex; F, purified subunit; G, fractions 40-60, Fig. 1; H, antiserum to the subunit. For each fraction an aqueous suspension of 10 mg/ml was made. Each well contained 7 µl suspension.

Table 1

KDO Content of Different Fractions[a]

Fraction	KDO %
Parent Complex	0.50
1st Peak (Fig. 3, Fractions 25-32)	1.5
Purified Subunit	-

[a]Results are expressed as the mean of five independent determinations.

DISCUSSION

We had previously shown that the antigenic complex of the Melvin strain of Neisseria gonorrhoeae is a protein composed of several subunits (6,7). In order to isolate its antigenic subunits, we incubated the complex in 8M urea at room temperature for four hours. The mixture was chromatographed on a Sepharose 6B column in 50 mM ammonium bicarbonate, pH 8.5 without 8M urea, and two peaks were obtained (Fig. 3). The second peak contained predominantly the subunit of molecular weight 66,000 daltons (Fig. 2C). It was purified from contaminants by affinity chromatography (Fig. 4, Fig. 2D). Treatment of the protein with dansyl chloride and subsequent thin layer chromatography on a polyamide sheet showed two spots, dansyl aspartic acid and dansyl amide, thus confirming the purity of the subunit. A smear under the band in SDS electrophoresis was observed with all preparations of the subunit and must represent a different form of the subunit under denaturing conditions; a low molecular weight smear was observed only above 50 µg. With different preparations we found the molecular weight of the subunit in the range of 66-68,000 daltons. The mobility of the subunit on both SDS gel electrophoresis and on Sepharose 6B chromatography (Fig. 3) was identical to that of BSA.

The omission of 8M urea from the eluent buffer of Sepharose 6B chromatography was effective in separating the subunit from the remaining complex. When 8M urea was included in the eluent buffer, the subunit was eluted in the void volume with the remaining complex. This suggests that in an unfolded state in the presence of 8M urea, the subunit has a high frictional ratio. When it is separated from the urea on the column, it presumably undergoes refolding and is included in the column. The remaining complex without the subunit, however, could not be dissociated even when chromatographed in the presence of 8M urea. The high amount of LPS in this complex could be responsible for preventing the dissociation of its subunits; only after the inclusion of detergent such as SDS could the dissociation of its subunits be achieved. We had previously reported that these subunits are bound by

hydrophobic interactions (6) and therefore need the presence of a detergent for their dissociation.

The results obtained from Ouchterlony immunodiffusion showed that the subunit is identical to the material present in the second peak of A-1.5M chromatography. This is possible if during the isolation of the complex some dissociation of the subunit takes place. Such dissociation was also observed during immunodiffusion of the parent complex. The absence of KDO, a component of LPS, from the subunit suggests that it is located either in the interior of the outer membrane or in the cytoplasm or cytoplasmic membrane. This data is consistent with the finding of Heckels (8) who has recently reported an outer membrane protein of molecular weight 60,000 daltons, devoid of LPS, from the parent strain P9 of Neisseria gonorrhoeae. However, in this study the antigenicity of the protein was not demonstrated. We have observed that the subunit is also present in other strains of the bacteria and shows antibody cross-reactivity. Because of its distribution, antigenicity and the absence of LPS, this protein may serve as an excellent vaccine candidate either alone or with other antigenic components isolated from the gonococcus. This report is the first demonstration of a new antigenic protein in Neisseria gonorrhoeae. Studies concerning the location of this protein in the bacteria will be published elsewhere.

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