ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1979, p. 336-340
0066-4804/79/09-0336/05$02.00/0
Vol. 16, No. 3

Comparison of the Penicillin-Binding Proteins of Different Strains of *Neisseria gonorrhoeae*

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Received for publication 26 June 1979

This study was undertaken to determine if the categorization of *Neisseria gonorrhoeae* strains into disseminated gonococcal infection (DGI) and non-DGI types was also paralleled by some common characteristic in their penicillin-binding proteins (PBPs). Fluorography of sodium dodecyl sulfate-containing polyacrylamide gels, on which the [^{14}C]penicillin-labeled PBPs had been separated, was used to visualize the PBPs. No common characteristic PBP for genital or DGI strains was observed. Apart from PBPs 1 and 4, which were observed in all cases, there was an apparently random distribution of other PBPs in both types of *N. gonorrhoeae*. It was concluded from these data that the penicillin susceptibility associated with most DGI strains of *N. gonorrhoeae* cannot be correlated with any specific changes in their PBP patterns.

Infections caused by *Neisseria gonorrhoeae* are mainly localized, producing symptoms limited to the genital region, rectum, or pharynx. In addition, a comparatively small number of cases have been characterized by symptoms involving skin and joints due to the spread of the infection. The latter type of disseminated gonococcal infection (DGI) is caused by strains of *N. gonorrhoeae* which are: (i) resistant to the bactericidal action of normal human serum, (ii) generally very susceptible to penicillin (minimal inhibitory concentration [MIC], ≤0.03 μg/ml), (iii) require arginine, hypoxanthine, and uracil for their growth (12), and (iv) can compete highly efficiently for iron (9). It was also demonstrated that serum resistance is associated with the possession of a principal outer membrane protein (POMP) of characteristic subunit molecular weight and antigenicity (5). The differential penicillin susceptibility of isolates from uncomplicated and disseminated gonococcal infections (DGI and non-DGI) led us to speculate that the DGI character might also be paralleled by the presence or absence of some specific penicillin-binding proteins (PBPs) in the inner membrane of *N. gonorrhoeae*. We have, therefore, used techniques similar to those described by Spratt (13) for *Escherichia coli* to examine the PBPs of a number of different DGI and non-DGI strains of *N. gonorrhoeae*.

MATERIALS AND METHODS

Strains. POMP serotyping, by enzyme-linked immunosorbent assay, was performed using purified gonococcal POMP from strain 7122 as described earlier (2, 4, 5). The serum bactericidal activities and auxotyping (5, 6, 11) of the *N. gonorrhoeae* strains used were performed in the laboratories of K. K. Holmes and T. M. Buchanan (at the Immunology Research Laboratory, U.S. Public Health Hospital, Seattle, Wash.), who also kindly supplied us with these strains. *E. coli* KN126 was generously supplied by Brian Spratt of the University of Leicester, Leicester, England.

The susceptibility to penicillin was determined using the agar dilution technique and is expressed as the MIC in micrograms per milliliter (12).

GC medium base (Difco 0289-02-0) containing 1% defined Kellogg supplement (8) was used as growth medium. The inoculated plates were incubated at 36.5°C in an atmosphere containing 5% CO_2 for 20 h.

The identification numbers of the strains used and their distinct properties are listed in Table 1.

Chemicals. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Serva (Heidelberg, F.R.G.). Dimethyl sulfoxide was from Fluka AG (Buchs, Switzerland).

[^{14}C]benzylpenicillin (specific activity, 51 to 53 mCi/mmol) was purchased from the Radiochemical Centre, Amersham, England. Small samples were dissolved in water and used within 24 h. Other β-lactam antibiotics were obtained commercially or synthesized in the chemistry department of this institute. All other reagents were of the highest quality available from Merck AG (Darmstadt, F.R.G.).

Methods. Preparation of crude membranes. *N. gonorrhoeae* strains were stored in liquid nitrogen passaged once on Kellogg agar (15) and grown on the same medium in 5% CO_2-air at 36.5°C for 20 h. The bacterial layer was scraped off the agar and washed with sterile 10 mM $NaPO_4$ buffer, pH 7.0. After centrifugation the pellet was resuspended in the same buffer containing 0.14 M β-mercaptoethanol. The bac-

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TABLE 1. *Characterization of the N. gonorrhoeae strains used*

Disseminated (D) or normal (N)/strain no.	Auxotype[a]	Complement-dependent serum killing[b]	POMP ELISA[c] test	Sodium penicillin G MIC (μg/ml)
D 1384	$A^- H^- U^-$	R	+	0.03
D 1939	$A^- H^- U^-$	R	+	0.03
D 1950	$A^- H^- U^-$	R	+	0.03
D 5296	$A^- H^- U^-$	R	+	0.06
D 5768	$A^- H^- U^-$	R	+	0.03
D 5785	$Pro^- A^- H^- U^-$	R	+	0.03
D 7122	$A^- H^- U^-$	R	+	0.03
N 5766	A^-	S	−	0.25
N 5767	A^-	S	−	0.125
N 6611	Prototroph	S	−	0.125
N 7189	Prototroph	S	−	0.25
N 8035	Prototroph	S	−	0.5
N 8038	$Pro^- A^-$	S	−	0.03

[a] Strain requires, for growth: A, arginine; H, Hypoxanthine; U, uracil; or Pro, proline. Prototroph, Prototrophic for proline, arginine, hypoxanthine, uracil, and methionine.
[b] R, Serum resistant; S, serum sensitive.
[c] ELISA, Enzyme-linked immunosorbent assay.

teria were then disrupted in this buffer, using three 1-min pulses of a Branson Sonifier with 1 min of cooling on ice between the pulses. Crude membranes were then prepared as described by Spratt (13) and stored at −70°C at a concentration of between 10 and 60 mg of protein per ml of 10 mM $NaPO_4$ buffer, pH 7.0. The protein concentration was determined by the technique of Wang and Smith (14).

Labeling of membrane proteins with [^{14}C]penicillin. A 0.2-mg portion of membrane protein was incubated with approximately 1.5×10^6 dpm of [^{14}C] benzylpenicillin in a total volume of approximately 40 μl. The sodium phosphate concentration was raised to about 75 mM by the addition of 100 mM sodium phosphate buffer, pH 7.0. The incubation period was 10 min at 30°C. A 5-mg amount of unlabeled benzylpenicillin was then added, and the [^{14}C]penicillin/membrane-protein complexes were stabilized and solubilized by the addition of 20 μl of 5% Sarkosyl. After standing at room temperature for 20 min the mixture was centrifuged at $100,000 \times g$ for 40 min in the 50 Ti rotor of a Spinco ultracentrifuge at 15°C. The outer membrane material remained insoluble and was pelleted, leaving the inner membrane proteins in the supernatant. This was removed (ca. 40 μl) and mixed with 10 μl of a solution containing 5% (vol/vol) β-mercaptoethanol, 350 mM tris(hydroxymethyl)-aminomethane-hydrochloride (pH 6.8), 5% (wt/vol) sodium dodecyl sulfate, 43% (vol/vol) glycerol, and 0.007% bromophenol blue. After the mixture was heated to 90°C for 5 min, it was applied to the gel system described below.

Acrylamide gel electrophoresis. The system described by Laemmli and Favre (7) was used, with 12% (wt/vol) acrylamide and 0.32% (wt/vol) visacrylamide in the running gel. The stacking gel was 7% (wt/vol) acrylamide and 0.19% (wt/vol) bisacrylamide. The slab gels were 1.6 mm thick and 15 cm wide. Electrophoresis was carried out at a constant current of 6.5 mA/gel for 15 h.

Fluorography. After electrophoresis the gel was stained for 10 min with shaking at 60°C in 0.25% (wt/vol) Coomassie brilliant blue–45% (wt/vol) methanol–10% (vol/vol) acetic acid. It was then electrically destained for 15 min, using 4 A at 60 V. The stained gel was then photographed and saturated with PPO (2,5-diphenyloxazole) as described by Bonner and Laskey (1). After rehydrating in 5% (vol/vol) glycerol–1% acetic acid, the gel was dried between two sheets of cellophane on a Bio-Rad model 224 gel slab drier. By using cellophane on both sides of the gel it is possible to expose two presensitized X-ray films (8) simultaneously, thereby reducing the exposure time.

Prebinding reactions. Preincubation of the crude membranes with a test β-lactam leads to the partial occlusion of the normal sites available for [^{14}C]benzylpenicillin binding. This occlusion is visualized as missing bands when compared with the standard pattern obtained after fluorography.

RESULTS AND DISCUSSION

The validity of separating *N. gonorrhoeae* strains into DGI and non-DGI types is given further support by the biochemical parameters summarized in Table 1. Not only do all the disseminated strains exhibit resistance to a complement-dependent serum killing, but they also possess a characteristic POMP as demonstrated by the POMP enzyme-linked immunosorbent assay. In addition, the DGI strains generally show a much lower penicillin G MIC and have particular growth requirements. Since little is known about the PBPs of *N. gonorrhoeae*, it was thought possible that in addition to the characteristics summarized in Table 1, the DGI character might also be reflected in some similarity in the PBPs which would be characteristic of these strains. Without referring directly to DGI and non-DGI types of *N. gonorrhoeae*, Rodriguez and Saz (10) investigated the total penicillin-binding capacity of *Neisseria* strains

exhibiting high and low penicillin G MICs. These workers found that there was an inverse relationship between the MIC and the penicillin-binding capacity of a given strain, particularly when the cytoplasmic membranes were exposed to high concentrations (20 μg/ml) of penicillin. In the experiments described in this communication, however, no such clear-cut difference was detectable between strains having high and low penicillin G MICs. It should be pointed out, however, that the concentration of [^{14}C]penicillin used in these assays was approximately 0.35 μg/ml, about 1/50 of the concentration used by the other workers. Furthermore, as can be seen from the data presented in the figures, even within the group of strains which we used there are significant differences in the total binding capacity, which appear to be unrelated to the MIC.

The profiles of the PBPs of various *N. gonorrhoeae* strains are depicted in Fig. 1 and 2; in addition, the *E. coli* KN126 pattern is included in Fig. 1 (track 7) to enable a comparison between the better-studied *E. coli* PBPs and those of *N. gonorrhoeae*. The main common characteristic seen throughout the *Neisseria* strains so far examined is that they all possess a PBP similar to that designated no. 1 in *E. coli*. Most strains also appear to have a protein which migrates at or very near to the region of PBP type 4 of *E. coli*. The bands corresponding to types 2 and 3 vary enormously in intensity and location, and many strains appear to have a

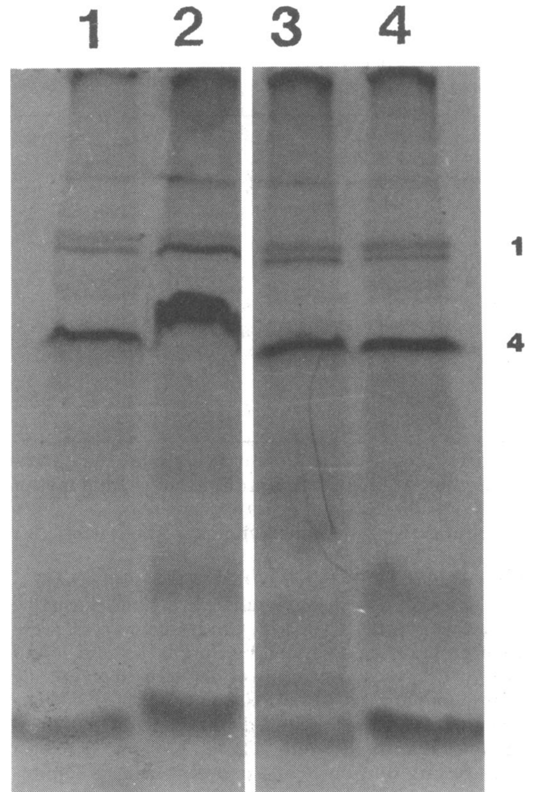

FIG. 2. *Fluorogram of the PBPs from track 1, DGI strain 1939; 2, DGI strain 1950; 3, non-DGI strain 6611; 4, non-DGI strain 8038.*

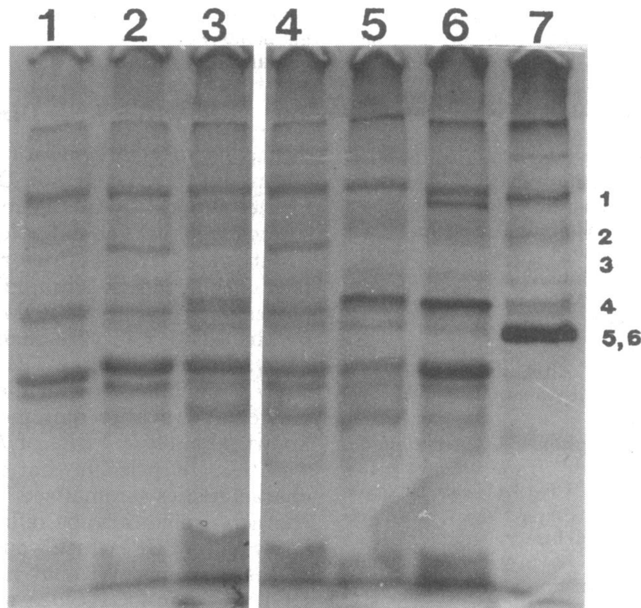

FIG. 1. *Fluorogram of the PBPs (see text) of: track 1, DGI strain 7122; 2, non-DGI strain 5766; 3, non-DGI strain 5767; 4, non-DGI strain 8035; 5, non-DGI strain 7189; 6, DGI strain 1384; 7, E. coli KN126.*

band which corresponds to the *E. coli* protein 5. The lower-molecular-weight PBPs, that is, those which migrate further into the gel, vary significantly in their appearance on fluorography, depending on the exact conditions of electrophoresis, the freshness of the acrylamide solutions, and other unknown factors. However, within any one particular gel these lower-molecular-weight proteins are always comparable. In experiments in which the membranes are pretreated with various antibiotics, no major differences in these low-molecular-weight binding proteins have so far been observed. It would thus appear that the significant PBPs are those of higher molecular weight corresponding approximately to that region of the gel occupied by *E. coli* proteins 1 to 6.

Pretreatment of membranes with β-lactam antibiotics produces characteristic changes in the binding patterns, some examples of which are depicted in Fig. 3. In this case the highly active new cephalosporin HR756 (cefotaxime) was used and produced the changes clearly visible in this figure. Of some interest is the fact that although the *N. gonorrhoeae* PBPs occurring in the *E. coli* band 1 region are virtually unaffected by cefotaxime preincubation, PBP 1 of *E. coli* itself has been completely eliminated. This clearly indicates that although these two protein types have similar physical characteristics, as shown by gel analysis, they may have very different physiological functions in the cytoplasmic membrane. The PBP corresponding to the position of *E. coli* protein 4 is totally eliminated in the two *Neisseria* strains by this preincubation with cefotaxime, whereas protein 4 of *E. coli* itself is only reduced in intensity. *E. coli* proteins 5 and 6, which frequently run together and appear as one band, are resolved clearly on this gel into two bands. In this case treatment with cefotaxime has reduced the penicillin-binding capacity of protein 6, which now appears as a much lighter band in the gel of the treated sample. By extending this type of study many interesting interspecies similarities can be deduced.

The data presented in Fig. 1 and 2 show that within the non-DGI class of *N. gonorrhoeae* there is a wide distribution of PBP profiles. The same can be said for strains in the DGI class. It must be concluded, therefore, that the DGI character is not reflected in the cytoplasmic membrane by the characteristic presence or absence of any particular PBP. Thus, the DGI character is predominantly determined by cell membrane characteristics such as the appearance of a dif-

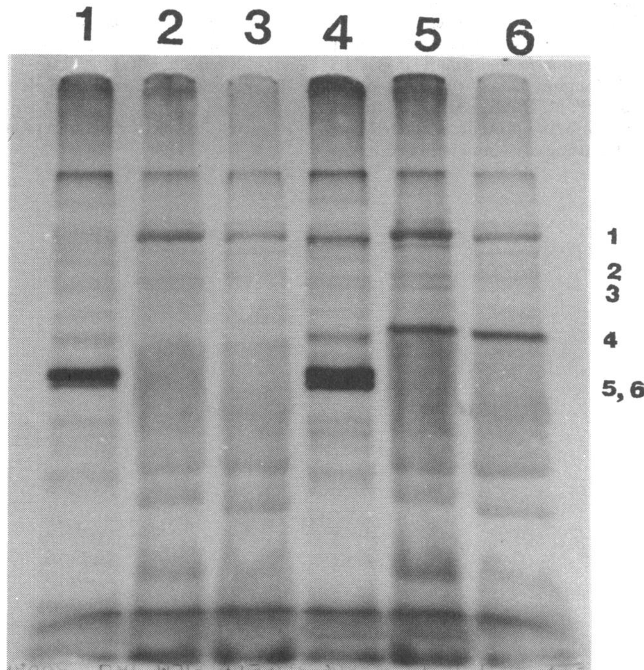

FIG. 3. *Effect of incubating crude membranes with cefotaxime on the subsequent [^{14}C]penicillin-binding pattern. Track 1, E. coli KN126; 2, N. gonorrhoeae 1384; 3, N. gonorrhoeae 7189, all pretreated with cefotaxime. Ten microliters of a 0.25-mg/ml solution of cefotaxime was used per 50-µl reaction volume. Tracks 4, 5, and 6 are the corresponding non-pretreated controls.*

ferent POMP, outer membrane changes which result in serum resistance, and cytoplasmic changes which result in the strain having particular nutritional requirements. Furthermore, as a result of these or other unknown changes in the outer membrane, the DGI strains are much more permeable to penicillin and other β-lactam antibiotics. Since so many outer membrane changes have been associated with the DGI character and since it has been shown here that the PBPs do not undergo characteristic changes in the DGI strains, it can be safely concluded that the DGI character is predominantly a consequence of outer membrane modification.

ACKNOWLEDGMENTS

We thank E. Bauer, C. Vogel, and H. Grasmuk for technical assistance and H. Gäth for help in preparing the manuscript.

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