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Properties of Penicillin-Binding Proteins in *Neisseria gonorrhoeae*

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The properties of penicillin-binding proteins (PBPs) of *Neisseria gonorrhoeae* were studied by comparing PBPs of clinical isolates of different penicillin susceptibility and by putting various β-lactam antibiotics in competition with radioactive penicillin for PBP binding. Apparent molecular weights of the three major PBPs found were 87,000 (PBP 1), 59,000 (PBP 2), and 44,000 (PBP 3). Relative penicillin resistance was associated with decreased binding to PBP 2 and, to a lesser extent, to PBP 1. Cephaloridine and benzylpenicillin, which produced spheroplasts at minimal inhibitory concentrations, bound to all three PBPs. In contrast, antibiotics which produced a majority of enlarged but apparently intact cells bound only to PBP 2 (mecillinam) or to PBPs 2 and 3 (cephalexin) at their minimal inhibitory concentrations.

The gram-negative rod *Escherichia coli* has seven penicillin-binding proteins (PBPs) associated with its inner membrane (20, 21). Some of these PBPs have been identified either with penicillin-sensitive enzymes involved in peptidoglycan synthesis (6, 12, 22, 23) or, when enzyme specificity remains undefined, with a process such as cell septation or cylindrical growth (21, 22). *Neisseria gonorrhoeae*, a gram-negative coccus, possesses PBPs as well (15, 16). Little is known, however, of the functions of individual gonococcal PBPs. Further information on gonococcal PBPs is of interest not only because of the shape and bidimensional growth pattern of the organism (25), but also because of the gradual decrease in degree of penicillin susceptibility shown by the majority of strains encountered over the past several years (18, 24).

In this study, I examined some properties of gonococcal PBPs by comparing PBPs of strains with different penicillin susceptibilities and by putting other β-lactam antibiotics in competition with radioactive penicillin for PBPs. It was found that *N. gonorrhoeae* had three major PBPs. Strains varied in the amount of penicillin binding to PBPs 1 and 2 but not to PBP 3. Although PBP 3 bound some β-lactam antibiotics at concentrations below their minimal inhibitory concentrations (MICs), PBPs 1 and 2 appeared to be more important targets of β-lactam action in *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains and conditions of growth. *N. gonorrhoeae* FA19 was provided by P. F. Sparling, University of North Carolina, Chapel Hill (4). Strains LP53 and KH6305 were provided by L. W. Mayer, Rocky Mountain Laboratories, Hamilton, Mont. LP53 was a protrophic urethral isolate which did not produce β-lactamase, and KH6305 was a disseminated infection isolate which required arginine, hypoxanthine, and uracil for growth (14).

Nonpiliated, transparent (T4) colonies were picked from plates of clear typing media of James and Swanson (7) after overnight incubation in 5% CO_2 at 37°C. The cells were grown at 37°C in a shaking water bath at GCH broth (13, 14), which was supplemented with 5 ml of 8.4% $NaHCO_3$ per liter. Cell growth was monitored by measuring the turbidity at 600 nm with a Beckman spectrophotometer. The generation times were 50 min for FA19 and LP53 and 69 min for KH6305. Exponentially growing cells were used in all experiments. An optical density of 0.5 was found to be approximately 5×10^8 colony-forming units per ml.

Antibiotics. [^{3}H]benzylpenicillin, ethylpiperidinium salt ([^{3}H]penicillin), with a specific activity of 31 Ci/mmol, was the generous gift of E. Stapley and P. Cassidy, Merck & Co., Inc., Rahway, N.J. [^{14}C]benzylpenicillin (30 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. Mecillinam was kindly provided by R. Cleland, Hoffmann-La Roche, Inc., Nutley, N.J. Cephaloridine, cephalexin, and the potassium salt of benzylpenicillin were obtained from Eli Lilly & Co., Indianapolis, Ind.

MIC. Tubes of broth containing serial twofold dilutions of antibiotic were inoculated with exponentially growing bacteria to a final concentration of 10^5 colony-forming units per ml. After 24 h of incubation at 37°C, the MIC was the lowest concentration which prevented turbid growth.

Membrane preparation. Cells were harvested from 3-liter cultures when the turbidity reached an optical density of 0.5. Before centrifugation ($5,000 \times g$ for 10 min), the cells were chilled, and $MgCl_2$ was added to a final concentration of 10 mM to minimize

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autolysis. The cell pellet was suspended in cold proteose-peptone-saline diluent (2) with 20 mM $MgCl_2$. All further manipulations were carried out at 4°C. Sonication was performed in the presence of 0.5% 2-mercaptoethanol with a Branson Sonifier with three pulses of 30 s each and with cooling intervals of 60 s. Centrifugation at 8,000 × *g* for 20 min removed unbroken cells. Cell membranes were pelleted out of the supernatant by centrifugation at 20,000 × *g* for 60 min. These membranes were washed with an equal volume of 50 mM sodium phosphate buffer, pH 7.0. Membranes were suspended in phosphate buffer at a protein concentration of 6 mg/ml, frozen in liquid nitrogen, and stored at −76°C. The yield of isolated membranes from a 3-liter culture was 10 to 20 mg of protein.

Binding of labeled penicillin to membranes and competition with various β-lactam antibiotics. The binding method was a modification of that used by Spratt (20). Samples (100 μl each) of a thawed membrane preparation were mixed with 10 μl of [^{3}H]penicillin in serial twofold dilutions. In the competition experiments, one of the various unlabeled β-lactam antibiotics or the phosphate buffer was incubated with the membrane preparation for 10 min at 37°C before the addition of [^{3}H]penicillin to a final concentration of 2.5 nmol/ml. After 10 min of incubation of membranes with [^{3}H]penicillin at 37°C, 10 μl of 4% sodium lauroyl sarcosinate (Sarkosyl) was added, followed by 5 μl of unlabeled benzylpenicillin (120 mg/ml). After 20 min of incubation at room temperature, the Sarkosyl-insoluble fraction was removed by centrifugation at 128,000 × *g* for 15 min in a Beckman Airfuge. A 100-μl portion of the supernatant, which predominantly contained inner membrane proteins, was added to 50 μl of gel sample buffer (20). To study binding to the outer membrane, the Sarkosyl-insoluble pellet was reextracted with 150 μl of 0.4% Sarkosyl, pelleted by centrifugation at 128,000 × *g* for 15 min, and suspended in 100 μl of phosphate buffer. This suspension was added to 50 μl of gel sample buffer.

Binding of labeled penicillin to PBPs in whole cells. Broth cultures were grown until the optical density reached 0.5. A 1-ml sample of culture was mixed with 100 μl of dilutions of [^{3}H]penicillin and incubated for 10 min in a water bath at 37°C. Immediately after the addition of 20 μl of unlabeled benzylpenicillin (120 mg/ml) and 20 μl of 1 M $MgCl_2$, the cell suspensions were placed on ice for 10 min. After 2 min of centrifugation in a Beckman Microfuge and decantation of the supernatant, the pelleted cells were resuspended in a solution containing 100 μl of phosphate buffer and 50 μl of gel sample buffer. This mixture was kept at room temperature for 20 min and agitated every 5 min before electrophoresis.

Gel electrophoresis and detection of PBPs. PBPs were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8). The concentrations of acrylamide and *N,N'*-methylenebisacrylamide were 10 and 0.13%, respectively, in the separating gel and 6% and 0.08%, respectively, in the stacking gel. Twenty microliters of 2-mercaptoethanol was added to the samples immediately before they were heated for 5 min in a boiling water bath. Enhance (New England Nuclear Corp., Boston, Mass.), a water-soluble fluor, was used for fluorography. Dried gels were exposed to presensitized Kodak X-Omat R film at −76°C (9). The exposure time was 1 to 3 days for membrane preparations and 3 to 5 days for whole cells. The amount of binding of [^{3}H]penicillin to PBPs was estimated by examining the X-ray film bands with a Joyce-Loebl microdensitometer. Integration of peaks was performed with the aid of a Zeiss MOP 3 image analyzer.

In these experiments, a PBP was defined as saturated when the activity of the emitting band, i.e., the area under the densitometry peaks, did not increase with higher concentrations of labeled penicillin or with longer incubation times. The 50% binding and 50% competition concentrations of labeled and unlabeled antibiotic were those values which would result in bands with densitometry peaks with one-half the area of peaks at saturation concentrations of [^{3}H]penicillin.

RESULTS

Binding of [^{3}H]penicillin to PBPs of *N. gonorrhoeae*. Figure 1 shows the PBPs of FA19 in both isolated membranes and whole cells. When [^{14}C]benzylpenicillin was used in conjunction with the fluorography method of Bonner and Laskey (1), identical results were obtained

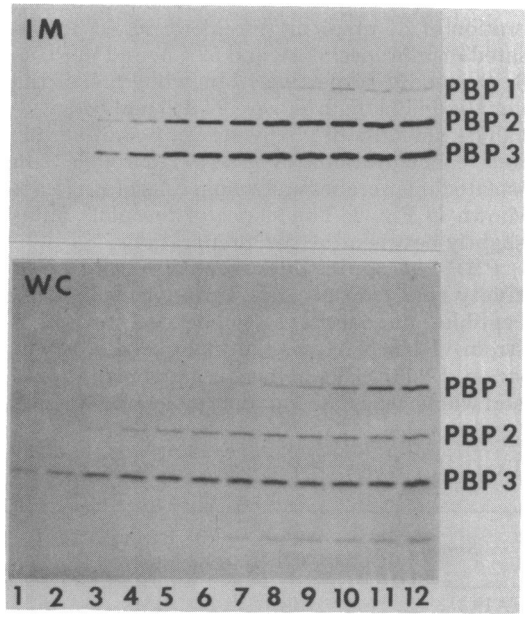

FIG. 1. *Fluorograms of PBPs of N. gonorrhoeae FA19 in isolated membranes (IM) and whole cells (WC). Electrophoresis was from the top to bottom. The distances to which the tracking dye ran into the separating gel were 8.4 cm for IM and 9.3 cm for WC. The following concentrations (nanomoles per milliliter) of [^{3}H]penicillin were incubated with preparations: 1, 0.0012; 2, 0.0024; 3, 0.005; 4, 0.01; 5, 0.02; 6, 0.04; 7, 0.08; 8, 0.15; 9, 0.31; 10, 0.62; 11, 1.25; and 12, 2.5.*

(data not shown).

The apparent molecular weights of the PBPs, when assessed by the addition of molecular weight standards to the gel, were 87,000 (PBP 1), 59,000 (PBP 2), and 44,000 (PBP 3). An additional band representing a protein with an apparent molecular weight of 34,000 was seen in the whole-cell preparation but not in the Sarkosyl-soluble (inner membrane) fraction of membranes. No bands were seen when the Sarkosyl-insoluble (outer membrane) fraction was examined by gel electrophoresis.

No additional PBPs were detected when the incubation period was varied between 30 s and 30 min. Prior incubation of membranes with 4 nmol of unlabeled benzylpenicillin per ml prevented the binding of [^{3}H]penicillin to PBPs 1 through 3. To assess the release of labeled penicillin from PBPs, a 1,000-fold excess of unlabeled penicillin was added to the reaction mixture of [^{3}H]penicillin-membranes reaction mixture after 10 min of incubation. There was no detectable release of label from any PBP when excess unlabeled penicillin was present for up to 80 min.

The percentages of total radioactive penicillin bound to individual PBPs of FA19 at a concentration of 2.5 nmol/ml were determined. In isolated membranes, 7, 41, and 52% bound to PBPs 1, 2, and 3, respectively. In whole cells, the corresponding figures were 24, 22, and 54%.

The amounts of [^{3}H]penicillin bound at various concentrations by PBPs 1 through 3 in isolated membranes and whole cells of FA19 are shown in Fig. 2. The slopes of the plots differ slightly between the two preparations.

PBPs of penicillin-susceptible and relatively resistant strains. In the penicillin-susceptible disseminated gonococcal infection strain, KH6305, and the relatively resistant strain, LP53, PBPs in both isolated membranes and whole cells had apparent molecular weights identical to those of FA19 (data not shown). In Table 1, the binding of [^{3}H]penicillin to PBPs in all three strains is compared. Whereas the amount of binding of [^{3}H]penicillin to PBPs 1 and 2 varied, binding to PBP 3 did not. LP53, which was 64- and 16-fold more penicillin resistant than KH6305 and FA19, respectively, bound between 10- and 20-fold less radioactive penicillin to PBP 2 in isolated membranes than the

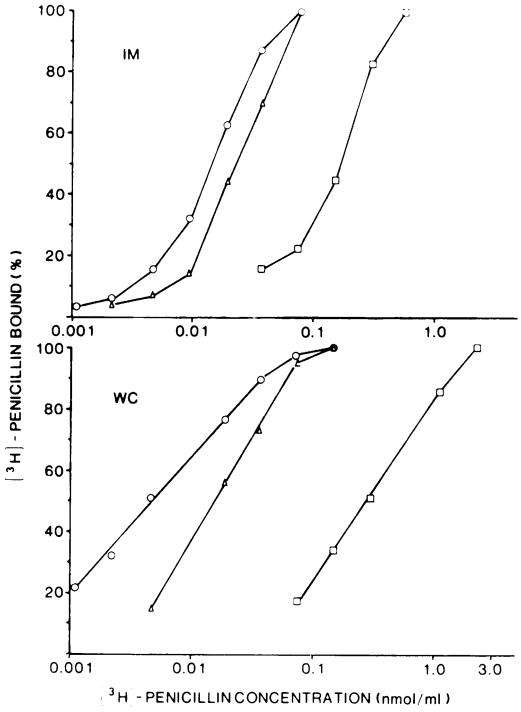

FIG. 2. *[^{3}H]penicillin binding to PBP 1 (□), PBP 2 (△), and PBP 3 (○) of N. gonorrhoeae FA19 in isolated membranes (IM) and whole cells (WC). The extent of binding to individual PBPs was measured by densitometry of fluorograms as described in the text.*

TABLE 1. *[^{3}H]penicillin concentrations required to give 50% binding of individual PBPs of N. gonorrhoeae FA19, LP53, and KH6305*

Strain	MIC (nmol/ml)	Binding prepn	Concn (nmol/ml) of [^{3}H]penicillin required to give 50% binding to:		
			PBP 1	PBP 2	PBP 3
FA19	0.04	IM[a]	0.17 (±0.04)[b]	0.023 (±0.004)	0.015 (±0.004)
		WC[c]	0.3	0.015	0.005
LP53	0.7	IM	0.6	0.4	0.01
		WC	1.0	0.6	0.004
KH6305	0.01	IM	0.3	0.04	0.01
		WC	0.3	0.02	0.004

[a] IM, Isolated membranes.

[b] Mean value from three independent determinations ± standard error. Remaining values are from experiments performed on the three strains at the same time.

[c] WC, Whole cells.

other two strains did. When whole-cell preparations were used, the ratio of the 50% binding concentrations in LP53 to those in the penicillin-susceptible strains increased to 30 to 40 for PBP 2. The differences among the strains in PBP 1 binding were less marked. Between FA19 and KH6305, which was repeatedly fourfold more susceptible to penicillin than was FA19, there were no significant differences in PBP binding in either isolated membranes or whole cells. When the time of incubation for reaction mixtures containing whole cells was varied between 30 s and 20 min, there also were no significant differences between FA19 and KH6305 in binding of [^{3}H]penicillin.

Competition of β-lactam antibiotics for PBPs. Other β-lactam antibiotics were chosen for study because of their different morphological effects and patterns of PBP binding in *E. coli* (20, 21). In *N. gonorrhoeae*, two types of morphological responses were seen at the MICs (Fig. 3). Cephaloridine produced spheroplasts, which are shown forming from lysing bacteria. Mecillinam and cephalexin (data not shown) produced mainly greatly enlarged but apparently intact cells. Benzylpenicillin produced both enlarged cells and spheroplasts. Table 2 shows, for isolated membrane preparations, the results of the competition of β-lactam antibiotics for PBPs of FA19, the MICs for these compounds, and the predominant morphological effects after 90 min of incubation at the MICs. Penicillin and cephaloridine, the spheroplast producers, bound to all PBPs at concentrations near or below their MICs. Cephaloridine bound to a greater proportion of available PBP 1 than PBP 2 at its MIC; the reverse was true for penicillin. At their MICs, cephalexin bound to both PBP 2 and 3, whereas mecillinam bound only to PBP 2. Although 1.5 nmol of both cephalexin and mecillinam per ml inhibited by 50% the subsequent binding of [^{3}H]penicillin to PBP 2, there were differences in the apparent binding of these two antibiotics at higher and lower concentrations (Fig. 4). Mecillinam is remarkable for the gradual slope of the PBP 2 competition curve when compared with the curves of cephalexin and cephaloridine.

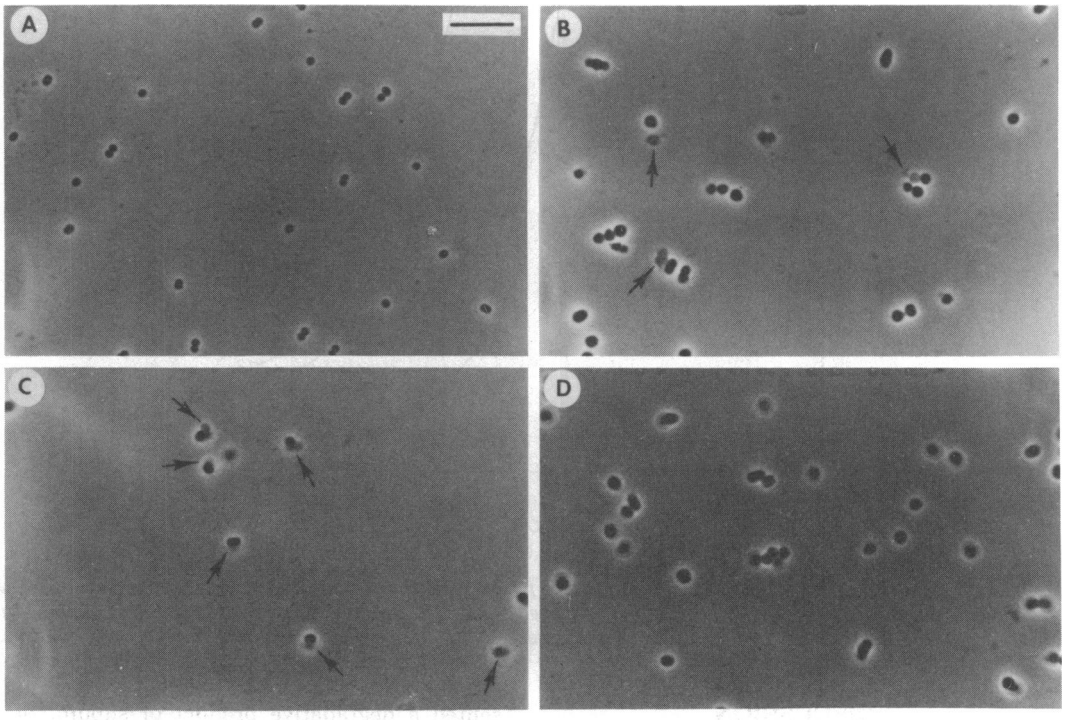

FIG. 3. *Morphological effects of β-lactam antibiotics on N. gonorrhoeae FA19. Exponentially growing cells in GCH broth (see text) were exposed to antibiotics at the MIC for 90 min. Cells were harvested in a Beckman Microfuge and resuspended in 0.1 volume of medium. Bacteria were examined immediately by phase microscopy with a Zeiss Photomicroscope II. (A) No antibiotic; (B) benzylpenicillin; (C) cephaloridine; (D) mecillinam. Bar = 10 μm. Arrows indicate spheroplasts or formation of spheroplasts.*

TABLE 2. *Concentrations of β-lactam antibiotics required to give 50% binding or competition, MICs, and morphological effects in N. gonorrhoeae FA19*

β-Lactam antibiotic	Concn (nmol/ml) of antibiotic required to give 50% binding to or 50% competition for:			MIC (nmol/ml)	Predominant morphological effect at MIC[a]
	PBP 1	PBP 2	PBP 3		
Penicillin[b]	0.2	0.02	0.015	0.04	Large cells and spheroplasts
Cephaloridine[c]	4	10	0.06	5	Spheroplasts
Cephalexin[c]	240	1.5	1.7	1.5	Large cells
Mecillinam[c]	130	1.5	70	1.5	Large cells

[a] Abnormal bacterial form(s) observed in 50 or more cells among 200 counted cells (methods as in legend to Fig. 3).

[b] Concentration of [^{3}H]penicillin which bound 50% of available PBP in isolated membranes (50% binding).

[c] Concentration of antibiotic which reduced by 50% the subsequent binding of [^{3}H]penicillin to PBP in isolated membranes (50% competition).

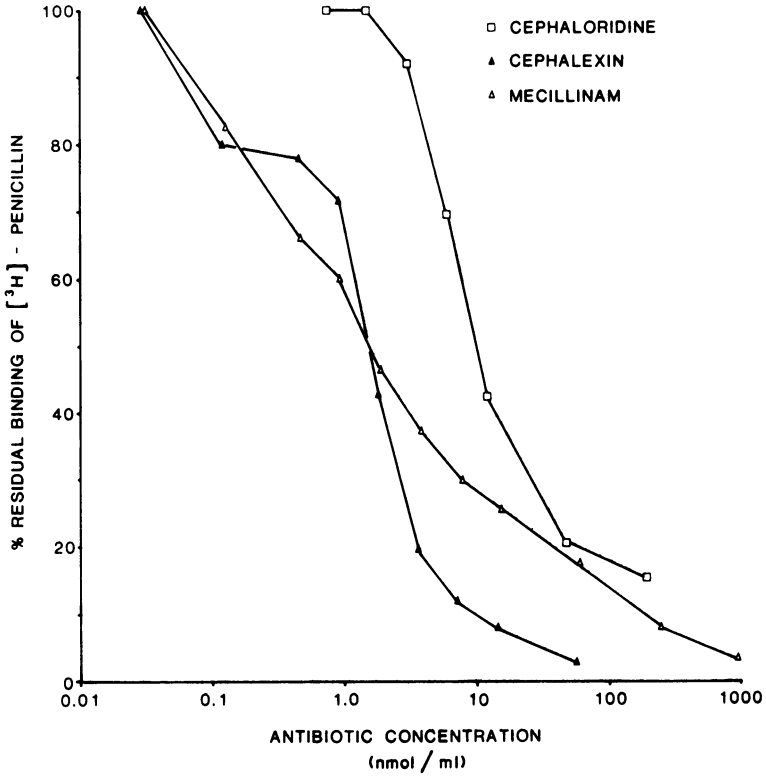

FIG. 4. *Competition of β-lactam antibiotics with [^{3}H]penicillin for binding by PBP 2. Samples of membranes of N. gonorrhoeae FA19 were preincubated with increasing concentrations of cephaloridine, mecillinam, and cephalexin. The amount of PBP still accessible to [^{3}H]penicillin (percent residual binding) was measured by procedures described in the text.*

DISCUSSION

This study demonstrated three major PBPs in *N. gonorrhoeae*. The nature of the fourth band, which was only seen in whole-cell preparations, remains unknown. It may have represented a degradative product or subunit of a higher-molecular-weight PBP. Alternatively, it may have been an inner or outer membrane protein that became, in isolated membranes, inaccessible to penicillin or altered in its penicillin affinity.

Rodriguez and Saz found several more emitting bands upon gel electrophoresis of membrane fractions in their study of penicillin binding in gonococci (16). These investigators used concentrations of labeled penicillin much in excess of the MICs of many of the organisms studied. I have produced such extra bands by using penicillin concentrations severalfold higher than those required to saturate PBPs 1, 2, and 3 (unpublished data). Nolan and Hildebrandt also found more than three bands in their examination of gonococcal PBPs with membranes prepared directly from overnight plate cultures (15). Under these conditions, autolysis may have been more pronounced than it was in broth cultures harvested during exponential growth. Additional bands may represent nonspecific binding of penicillin to fragments of proteolysed PBPs or other proteins.

This study has also shown variation in the amount of penicillin bound by two individual PBPs in the strains examined. The findings of decreased binding to PBP 2 and, to a lesser extent, to PBP 1 in a relatively resistant strain agree with results recently reported (T. J. Dougherty, A. E. Koller, and A. Tomasz, Program Abstr. Eur. Mol. Biol. Organ. Workshop Genet. Immunobiol. Pathogenic *Neisseria*, Hemavan, Sweden, 1980, abstr. no. 8). These investigators also found three major PBPs as well as decreased binding in a resistant strain to the PBPs they designate as 1 and 2. Both the present report and that of Dougherty et al. confirm and extend the findings of Rodriguez and Saz (16). Scudamore also speculated that PBPs may be altered in resistant gonococci (17). The gonococcus joins the pneumococcus on the list of bacterial species for which one mechanism of resistance in clinical isolates is altered binding to the target (5, 26).

However, other mechanisms may also be involved in gonococcal penicillin resistance that is not β-lactamase mediated. An alteration in the cell envelope, such as that described by Sparling and co-workers (4, 19), may contribute to resistance as well. The inability to explain the consistently greater susceptibility of those disseminated gonococcal infection strains examined in both this study and in the study of Nolan and Hildebrandt (15) also suggests that other factors are involved.

The competition of various β-lactam antibiotics with radioactive penicillin for PBPs has been useful for studying the functions of individual PBPs (20). Nevertheless, when unlabeled antibiotics are employed, certain alternative explanations for results cannot be entirely excluded. One possibility is that there are PBPs which form extremely short-lived, and therefore undetectable, complexes with a β-lactam antibiotic. There may also be targets for β-lactam antibiotics other than those which bind labeled penicillin. Mindful of these potential limitations, I can make some interpretations.

The predominant morphological responses by gonococci to β-lactam antibiotics were enlarged, spherical cells and cells forming spheroplasts. Lorian and Atkinson (10) and Westling-Häggström et al. (25) found similar responses. Penicillin and cephaloridine bound to all three PBPs and produced many spheroplasts under the conditions employed. On the other hand, cephalexin and mecillinam, which produced a majority of large and apparently intact cells, did not detectably bind to PBP 1 at concentrations near their MICs. PBP 1 of *N. gonorrhoeae* resembles PBP 1b of *E. coli* in cephaloridine affinity and association with spheroplast formation (20, 21). Gonococcal PBP 1 may, like *E. coli* PBP 1b (23), be involved in cell wall expansion and in crosslinking peptidoglycan side chains.

The similar morphological effects and degree of binding to PBP 2 shown by mecillinam and cephalexin in *N. gonorrhoeae* contrast with the different effects and affinities of these two drugs in *E. coli* (20, 21). In *E. coli*, mecillinam produces spherical cells and binds only to PBP 2, whereas cephalexin produces filamentous cells and binds to PBP 3 at low concentrations. In view of the difference in growth patterns between *E. coli* (3) and *N. gonorrhoeae* (25), gonococcal PBP 2 may serve a function in gonococcal cell division that is shared by PBP 2 and PBP 3 in *E. coli*.

PBP 3 of *N. gonorrhoeae* bound the greatest proportion of labeled penicillin and also was 50% bound at concentrations of cephaloridine that were considerably below the MIC. However, there is evidence that, like PBPs 5 and 6 of *E. coli* (12), PBP 3 of *N. gonorrhoeae* is not an important target of β-lactam action. The undiminished binding to PBP 3 in a relatively resistant strain, which probably evolved from a penicillin-susceptible strain (11, 19), suggests either that a mutation in the putative PBP 3 gene resulting in penicillin resistance through decreased binding is very deleterious to the organism or that PBP 3 is not a critical target. Further support of the latter hypothesis is the fact that mecillinam bound detectably only to PBP 2. This suggests that binding to PBP 3 or 1 is not necessary for effective β-lactam action.

These studies have revealed a number of properties of gonococcal PBPs. The relatively low number of PBPs found in this species, as well as the continued importance of β-lactam antibiotics in therapy of gonococcal infections, would

seem to make *N. gonorrhoeae* a very suitable organism in which to pursue further both the biological implications and the pharmaceutical applications of PBPs.

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