ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1987, p. 178–182
0066-4804/87/020178-05$02.00/0

Vol. 31, No. 2

Alterations in Peptidoglycan of *Neisseria gonorrhoeae* Induced by Sub-MICs of β-Lactam Antibiotics

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Received 23 June 1986/Accepted 12 November 1986

Exposure of *Neisseria gonorrhoeae* to sub-MICs of selected β-lactam antibiotics caused distortion of normal cell morphology. Analysis of the peptidoglycan indicated that the cells were accumulating increased quantities of disaccharide pentapeptide in their cell walls. The O-acetylated form of the disaccharide pentapeptide was not detected among the major peaks. The correlation of antibiotic binding to gonococcal penicillin-binding protein 2 and accumulation of non-O-acetylated disaccharide pentapeptide suggested an explanation for the previously observed relationship of penicillin-binding protein 2 and O-acetylation of peptidoglycan.

The penicillin-binding proteins (PBPs) are a group of enzymes that participate in the terminal steps of peptidoglycan assembly (19, 21). Several approaches have been used to define the roles of individual PBPs in the assembly process. These include in vitro systems with purified PBPs and lipid-linked peptidoglycan precursors and systems in which permeabilized cells are incubated with nucleotide precursors. The former procedure is very laborious, requires exacting reaction conditions, and yields little product (14). The latter method requires harsh organic solvents for permeabilization and may be susceptible to significant artifacts (9, 15). Although both methods have yielded useful results, we have chosen an approach that involves treating intact, logarithmically growing *Neisseria gonorrhoeae* cultures with selected β-lactam antibiotics. These antibiotics were used at concentrations that allowed continued peptidoglycan synthesis under conditions of selective PBP inhibition. After a period of wall synthesis, the peptidoglycan was isolated and analyzed with reverse-phase high-performance liquid chromatography (RP-HPLC). This technique, which was previously applied to *Escherichia coli* (7, 11) and *N. gonorrhoeae* (1, 5), reveals an unexpectedly complex peptidoglycan structure. In the case of the gonococcus, the complexity is further increased by the presence of O-acetyl groups on the disaccharide peptide units (2, 4).

In the present study, we found a correlation between binding to gonococcal PBP 2 and a major increase in incorporation of disaccharide pentapeptide into insoluble peptidoglycan. This marked increase was limited to the non-O-acetylated form of this pentapeptide subunit. These results may explain the previous observations in the literature with regard to the effects of β-lactam antibiotic on the degree of peptidoglycan O-acetylation (2, 4).

MATERIALS AND METHODS

Bacterial strain and growth conditions. *N. gonorrhoeae* FA19101 (*penA*) was used throughout this work (4). The strain was maintained by daily transfer on GCBA agar supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Colony morphology was p^- (pilus negative) and transparent by the criteria of Swanson (20). MICs of antibiotics were determined by agar plate dilutions as described previously (18).

Bacteria were grown in gonococcal broth with IsoVitaleX and 420 μg of $NaHCO_3$ per ml at 37°C with aeration (4). Growth was monitored in a Sequoia-Turner model 340 spectrophotometer at 610 nm. Antibiotics were added at low absorbance values (0.05 absorbance unit), and growth was continued for 3.5 generations (0.6 absorbance unit; 249 μg [dry weight] per ml of cells). Cells for large-scale peptidoglycan preparations were harvested after rapid chilling in an alcohol-ice bath.

PBPs. Samples of 1 ml were removed from the liquid cultures just before harvesting and incubated in saturating concentrations (2.0 μg/ml) of [^{3}H]benzylpenicillin (25.4 Ci/mmol; Merck & Co., Inc., Rahway, N.J.) to determine residual PBPs. After 15 min, cells were prepared, and PBPs were analyzed as previously described (4). The amount of labeled penicillin bound to individual PBPs was determined by densitometry on Helena Quick Scan Jr. (Helena Laboratories, Beaumont, Tex.) interfaced with a Rockwell AIM-65 microcomputer.

Peptidoglycan analysis. Gonococcal peptidoglycan was isolated from cells by hot sodium dodecyl sulfate extraction exactly as described previously (5). A small sample of each preparation was subjected to acid hydrolysis and amino acid analysis as a purity check. The remainder of the sample was digested with 25 μg of *Streptomyces globisporus* muramidase (Miles Scientific, Naperville, Ill.) overnight at 37°C in 20 mM sodium phosphate buffer (pH 6.5).

Muramidase-digested peptidoglycan was analyzed in the RP-HPLC system as described previously (5). The RP-HPLC profiles obtained were virtually identical to those previously described with this system, although absolute retentions were somewhat different from that reported due to slight buffer variations and column aging. The total amount of peptidoglycan injected in each analysis was 80 to 100 μg.

The presence of O-acetyl groups was determined by treatment at pH 10 for 6 h, which results in an 80 to 90% decline in areas of O-acetylated muropeptide peaks (2, 5). Assignment of cross-linkage (monomers, dimers, trimers) was by size fractionation on Sephadex columns before HPLC separation (5). Muropeptides with identical (ca. 1% variance) retention times in several analyses were manually collected and pooled. The material was dried in a Savant Speed Vac centrifugal concentrator (Savant Instruments,

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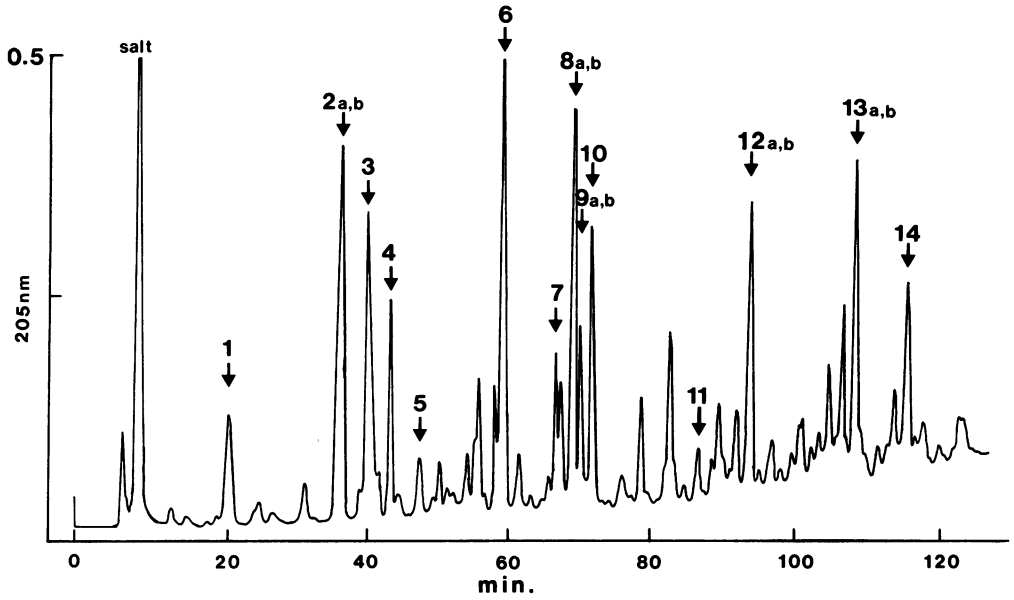

FIG. 1. Preparative separation of gonococcal peptidoglycan components. Approximately 100 μg of muramidase-digested peptidoglycan was injected into the RP-HPLC system, and the major muropeptides were collected. Numbered peaks correspond to the chemical analyses presented in Table 1. Detection was at 205 nm with the detector at 0.5 absorbance unit, full scale.

Hicksville, N.Y.). The analytical separation requires the presence of 50 mM phosphate buffer, and the pooled muropeptides were therefore heavily contaminated with salt.

The salt was removed in a second chromatographic step by using volatile mobile phases. The dry samples were dissolved in 500 μl of HPLC-grade water. Each peak was individually desalted by reinjection into an HPLC system that differed from the analytical mode. In this case, elution was with a linear gradient of 0.05% trifluoroacetic acid in water to 0.05% trifluoroacetic acid in water with 50% (vol/vol) acetonitrile over 20 min. The column was a Merck LiChrosorb RP-18 (5-μm particles, 0.4- by 250-mm column; EM Science, South Plainfield, N.J.). Excellent separations of salt and muropeptides were obtained. In addition, several of the muropeptides that eluted as single peaks in the first separation gave two peaks during the desalting chromatography. These were analyzed separately. The isolated peaks were again dried in the centrifugal evaporator, and 200 μl of 5.8 N HCl (Sequanal grade; Pierce Chemical Co., Rockford, Ill.) was added. Tubes were evacuated, sealed, and heated to 110°C for 4 h (10). The hydrolyzed samples were analyzed on a Durrum D-500 amino acid analyzer with detection by ninhydrin reaction.

RESULTS

Growth characteristics of antibiotic-treated cells. Previous studies have established that continued gonococcal growth and peptidoglycan synthesis occur when either PBP 2 or PBP 3 is almost totally bound with a β-lactam antibiotic (4). Inhibition of PBP 1 leads to rapid cessation of continued wall synthesis and growth (4). A number of β-lactam antibiotics were tested at subinhibitory concentrations; based on the PBP binding profiles, several were selected for further study.

Growth rates were determined by culture turbidity. The growth rate was slightly reduced from a 55-min doubling time to 70 min with piperacillin and cefotaxime at 0.25× MIC. Penicillin at 0.25× MIC caused only minimal changes to a doubling time of 60 min.

Cefotaxime and piperacillin were found to produce some subtle changes in normal cell morphology at 0.25× MIC (data not shown), whereas penicillin produced a modest swelling (13).

Analysis of peptidoglycan after antibiotic treatment. The high resolution of the RP-HPLC technique allowed the study of rather subtle disturbances in peptidoglycan metabolism in the presence of sub-MICs of antibiotics. The HPLC profile of an untreated control culture is shown in Fig. 1. The muropeptide peaks which were labeled were isolated, and amino acid analysis was performed as detailed in Materials and Methods (Table 1). The identities of disaccharide tripeptide (peak 1), disaccharide tetrapeptide (peak 2B), and bis-disaccharide components (peaks 10 and 12A) were confirmed, as were the identities of their O-acetylated counterparts (1, 5). In addition, several points are worthy of mention. One of the two peaks at 37 min, peak 2A, contained a molar quantity of glycine, similar to the situation reported in *E. coli* (7). A group of disaccharide dipeptides, all devoid of diaminopimelic acid, was found between 66 and 73 min (8). One of the peaks, 8A, also had equimolar quantities of glycine and alanine, with the glycine presumably on the glutamic acid (6).

Peptidoglycan was isolated from gonococci grown in the presence of sub-MICs of antibiotics for 3.5 generations. The profiles obtained by RP-HPLC differed significantly from the control. Figure 2 illustrates the muropeptides observed. The most striking feature was the increase in the peak at 48 min (Fig. 2, arrow) in the β-lactam antibiotic-treated cultures. This peak was isolated from gonococci grown in antibiotics and identified as the non-O-acetylated disaccharide pentapeptide. However, although the monomer fraction of gonococcal peptidoglycan is 55 to 60% O-acetylated (2, 4) and we examined all of the major peaks eluting in the monomer region, we did not observe a peak which increased that consisted of O-acetylated disaccharide pentapeptide. This was in spite of the fact that the disaccharide pentapeptide became the predominant monomer species, and the O-acetylated forms of disaccharide tripeptide (peak 4) and

TABLE 1. Composition of muropeptides of *N. gonorrhoeae*

Peak[a]	Retention time (min)	Amino acid composition[b]				O-acetylation[c]	Cross-linkage[d]
		Glu	Gly	Ala	DAP[e]		
1	22	1.0	–[f]	0.8	1.0	–	Monomer
2A	37	1.0	1.0	1.9	1.3	–	Monomer
2B	37	1.0	–	1.9	0.8	–	Monomer
3	41	1.0	–	1.0	0.0	–	Monomer
4	44	1.0	–	1.7	0.9	+	Monomer
5[g]	48	1.0	–	3.0	1.2	–	Monomer
6	60	1.0	–	2.1	1.4	+	Monomer
7	67	1.0	–	1.0	0.0	+	Monomer
8A	69	1.0	0.6	0.7	0.0	–	Monomer
8B	69	1.0	–	0.9	0.0	–	Monomer
9A	70	1.0	–	0.8	0.0	–	Monomer
9B	70	1.0	–	1.0	0.0	–	Monomer
10	72	1.0	–	1.0	1.0	–	Dimer
11[g]	84	1.0	–	2.4	1.2	–	Dimer
12A	93	1.0	–	1.5	1.3	–	Dimer
12B	93	1.0	–	1.6	1.0	+	Dimer
13A	109	1.0	0.3	1.7	1.3	–	Trimer
13B	109	1.0	–	1.8	1.3	+	Trimer
14	115	1.0	–	1.7	1.2	–	Trimer

[a] Peak numbers correspond to labels in Fig. 1.
[b] Ratios based on glutamic acid as standard (1.0).
[c] Determined by sensitivity to base hydrolysis (pH 10, 37°C for 6 h).
[d] Determined by prior size fractionation on coupled Sephadex G-50 and G-25 columns (5).
[e] DAP, Diaminopimelic acid.
[f] –, Trace amounts not integrated by the amino acid analyzer.
[g] These peaks were collected from a piperacillin-treated culture in which their quantities were amplified.

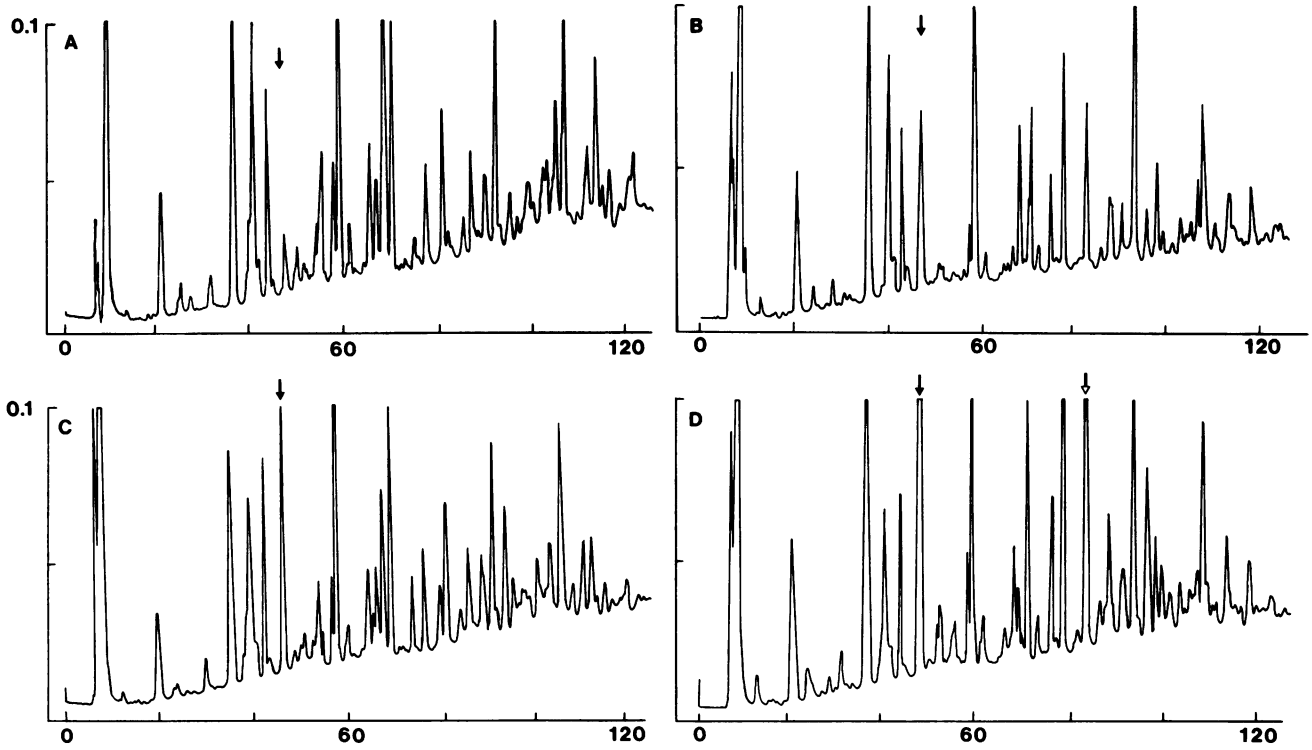

FIG. 2. RP-HPLC profiles of gonococci treated with β-lactam antibiotics. Illustrated are the muropeptides of (A) control cells and cells treated with (B) penicillin (0.015 μg/ml), (C) cefotaxime (0.002 μg/ml), and (D) piperacillin (0.0035 μg/ml). All antibiotics were present at 0.25× MIC. The position of disaccharide muramyl pentapeptide is indicated in all four samples by the solid arrow. The open arrow in panel D indicates the amplified component at 84 min. The detector was set at 205 nm and 0.1 absorbance unit, full scale. The time scale (abscissa) is in minutes.

TABLE 2. PBP binding of antibiotics

Antibiotic[a] (μg/ml)	% of PBP bound[b]			% Disaccharide pentapeptide[c]
	PBP 1	PBP 2	PBP 3	
Control (0)	0	0	0	1.2
Penicillin (0.015)	24.8	15.2	96.8	5.0
Cefotaxime (0.002)	13.3	62.7	22.4	7.0
Piperacillin (0.035)	6.7	90.1	37.2	11.4

[a] Antibiotics were present at 0.25× MIC.

[b] Numbers are averages of triplicate determinations. The percentage bound was determined by comparison of β-lactam-treated cells with equal mass (absorbance at 610 nm) of control cells, both treated with saturating [^{3}H]penicillin. Quantitation was by scanning fluorographs with a computing densitometer.

[c] Percentage of total peptidoglycan, obtained from HPLC system integrator.

tetrapeptide (peak 6) were readily found under these conditions. Examination of the PBP binding data for these experiments (Table 2) showed that the increased amounts of disaccharide pentapeptide correlated with the increasing degree of PBP 2 binding. Other changes were noted upon antibiotic treatment, although these were variable depending on the antibiotic used. The decline in the late-eluting peaks of highly cross-linked trimers and tetramers that was particularly acute in the case of penicillin was interesting, inasmuch as this compound showed significant PBP 1 binding. Prior evidence has suggested a role for PBP 1 in transpeptidation (4). Finally, the peak at 84 min, most notable in the piperacillin-treated cells (Fig. 2D, open arrow), was found to have a composition consistent with a bis-disaccharide dimer having one tetrapeptide side chain and one pentapeptide chain.

DISCUSSION

In the present study, the treatment of gonococci with sub-MIC (0.25× MIC) levels of antibiotics allowed continued cell growth and macromolecular synthesis. The RP-HPLC technique permitted a detailed analysis of the cell wall structure under these conditions. Exposure of gonococci to sub-MICs of selected β-lactam antibiotics yielded situations in which either PBP 3 (penicillin) or PBP 2 (cefotaxime and piperacillin) was significantly bound (Table 2). As found previously, increasing levels of PBP 1 binding led to a drastic reduction in peptidoglycan synthesis and subsequent cell lysis (4). In the case of PBP 3, we could not correlate binding to a change in the peptidoglycan profile. There did, however, appear to be a relationship between PBP 2 binding and increased amounts of disaccharide pentapeptide incorporated into the sodium dodecyl sulfate-insoluble cell wall fraction. Although we isolated and analyzed virtually all of the major peptidoglycan fragments generated by this method (Fig. 1 and Table 1), we did not find a corresponding major peak of O-acetylated disaccharide pentapeptide.

This finding suggests one plausible explanation for the effect of β-lactam antibiotics on O-acetylation of gonococcal peptidoglycan (4). High-affinity binding to PBP 2 would inhibit carboxypeptidase activity, leading to increased pentapeptide-containing subunits in the wall. O-acetylation is a postsynthetic modification of the gonococcal peptidoglycan (3, 12), and it is conceivable that pentapeptide subunits are poor substrates for this reaction. Thus the failure to process incorporated dissacharide pentapeptide to tetrapeptide via D,D-carboxypeptidase action could indirectly inhibit further the O-acetyl modification of the peptidoglycan. The D,D-carboxypeptidase activity would presumably reside in PBP 2. Thus, an immature form of cell wall would accumulate and may be responsible for the altered morphogenesis observed. Interestingly, Rosenthal et al. have observed increased levels of O-acetylation in gonococci treated with inhibitors of protein synthesis (17). In this case, there is a rapid rise upon chloramphenicol addition primarily in O-acetylation of new monomer subunits. Whether this means that the generation of processed muropeptide substrate for O-acetylation is optimal under these conditions remains to be demonstrated.

Other, more variable changes in peptidoglycan structure were also evident. The decrease in cross-linked peptidoglycan observed with penicillin can be attributed to PBP 1 binding, since this PBP has been implicated in transpeptidation reactions. It should also be noted that although results at 0.25× MIC are reported herein, identical qualitative changes were observed at 0.12× and 0.06× MIC, albeit to a lesser degree.

In the course of these investigations, considerable data on the amino acid compositions of peptidoglycan subunits were obtained (Table 1). These data should serve as a baseline for further chemical characterizations of gonococcal peptidoglycan components.

Clearly, the employment of RP-HPLC has significant potential in the study of peptidoglycan metabolism and β-lactam antibiotic action. Studies to date of sub-MIC effects on bacterial morphology or interaction with host cell components have not examined the influence of the antibiotics on the peptidoglycan (13, 16). The techniques described in this report have direct applicability to investigations in these areas.

ACKNOWLEDGMENTS

We thank Alexander Tomasz for critical reading and discussions. We also acknowledge the assistance of Maria Pospischil with amino acid analyses and of Maria Lipp and Margaret Geller with manuscript preparation.

This work was supported by Public Health Service grant AI20020 from the National Institutes of Health. J.F.G.-B. was the recipient of a Fulbright Fellowship from the Spanish Ministry of Education and Science.

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