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Effects of MICs and Sub-MICs of Antibiotics on Production of Capsular Polysaccharide of *Klebsiella pneumoniae*

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In the present study, we examined whether MICs and sub-MICs of antimicrobial agents belonging to two different classes, ciprofloxacin and ceftazidime, were able to influence the production and release of cell-associated and soluble (extracellular) capsular polysaccharide (CPS), respectively, in a heavily encapsulated strain of *Klebsiella pneumoniae* (B5055). Using a CPS-specific enzyme-linked immunosorbent assay, we found that the amount of cell-associated CPS increased in a dose-dependent manner by more than 10-fold under the influence of the MIC of ceftazidime and by more than 100-fold under the influence of the MIC of ciprofloxacin. The largest amounts of CPS were measured by using the MIC of either antibiotic substance. Electron microscopic studies showed that the diameter of the capsule was significantly increased compared with the diameter for untreated controls. Thus, both antimicrobial agents genuinely stimulated CPS production.

In vitro, antibiotic concentrations below the MIC (sub-MICs) have been shown to modulate the expression of various bacterial virulence mechanisms (1). Recent studies also have shown that the exposure of bacteria to sub-MICs of quinolones results in a significant alteration of cellular morphology and a disturbance of metabolic activity (3, 4, 7, 22), as well as increased susceptibility of bacteria to phagocytic killing by human neutrophils (20). Similar effects were described for cephalosporins (12). In the human body, the concentration of antimicrobial agents is often below the MIC (18). It is questionable, therefore, whether such sub-MICs of antimicrobial agents contribute to therapeutic effects. Sub-MICs of antimicrobial agents may affect the production of cell wall components, resulting in a quantitative reduction.

To study this question, we examined the capsule of *Klebsiella pneumoniae* because it is an important virulence factor (10) and because it is now possible to quantitate its amount directly by an enzyme-linked immunosorbent assay (ELISA) method (9, 24). We presumed that there may be a reduction of capsular polysaccharide (CPS) production under the influence of MICs and sub-MICs of several antimicrobial agents, as was suggested by others using indirect methods to investigate CPS production (12, 14, 27). In contrast to this hypothesis, we found an unexpected increase in CPS production in the presence of both of the antimicrobial agents used in the present study.

(This report is part of the M.D. thesis of C. Adamczik.)

MATERIALS AND METHODS

K. pneumoniae B5055 (O1:K2) was obtained from Ida Ørskov, Statens Serum Institut, Copenhagen, Denmark, and used throughout the study. The bacteria were grown in commercial brain heart infusion (BHI) broth (Hoffmann-La Roche, Grenzach-Wyhlen, Germany) at 37°C with gentle shaking (100 rpm). Log-phase cultures were adjusted densitometrically to the desired bacterial concentration, which was confirmed by plating serial 10-fold dilutions of the suspension on Trypticase soy agar (Oxoid, Basingstoke, United Kingdom).

Ciprofloxacin (Bayer, Leverkusen, Germany) and ceftazidime (Glaxo, Ware, United Kingdom) were obtained as powders of known potency and were diluted to the desired concentrations in sterile phosphate-buffered saline according to the manufacturers' instructions. MICs were determined according to National Committee for Clinical Laboratory Standards guidelines (11). The medium used was Mueller-Hinton broth (Oxoid), and the quality control organism was *Escherichia coli* ATCC 25922. The determination of the MICs was done by the broth microdilution method as described elsewhere (11). The chosen sub-MICs were three-quarters, one-half, and one-quarter of the MIC of ceftazidime and two-thirds, one-third, and 1/30 of the MIC of ciprofloxacin. After incubation of the bacteria in BHI for 4 h (37°C, 100 rpm), approximately 10^7 CFU was transferred into fresh BHI containing the desired concentrations of ciprofloxacin or ceftazidime and incubated again (37°C, 100 rpm). Control cultures were grown in BHI only, under the same conditions. At 0, 1, 2, 4, 6, 8, and 10 h, 4 ml of each culture was withdrawn and immediately placed on ice for subsequent analysis. A 1-ml aliquot was used for CFU determinations by plating serial 10-fold dilutions. Of the remaining 3 ml, 1 ml was used to determine total protein, 1 ml was used to determine cell-bound CPS, and 1 ml was used to determine free CPS. The last fraction was filter sterilized (pore size, 0.45 μm; Renner, Dannstadt, Germany) and stored at −20°C until further use. Extraction of cell-bound CPS as well as cell disintegration for total protein determination were done immediately after withdrawal of the samples. Each experiment was performed at least four times.

For the quantification of cell-bound CPS, bacteria were treated according to the method of Domenico et al. (5, 6). Samples were stored at −20°C until further use. Concentrations of K2 CPS were determined by means of a previously described ELISA method based on a K2-specific monoclonal antibody (9, 24).

For cell disruption prior to the determination of total bacterial cell protein, an aliquot of 1 ml was centrifuged (8,000 × *g*, 5 min) and the supernatant was removed. The pellet was washed in sterile normal saline and centrifuged again (8,000 × *g*, 5 min). After removal of the supernatant, the pellet was resuspended in a lysing solution (distilled water containing 2% [wt/vol] sodium dodecyl sulfate [Bio-Rad, Richmond, Calif.]) and incubated at 65°C for 10 min. The cell lysates were then stored at −20°C until further use. Previous experiments showed that this procedure resulted in a lowering of the CFU counts from 10^7/ml to approximately 10^3/ml. In addition, densitometry performed after lysis showed virtually no difference between the samples and the lysing solution (data not shown), indicating an almost 99.99% lysis of the bacteria. Lysis with a French pressure cell press (SLM Instruments Inc., Urbana, Ill.) at 2,000 lb/in^2 for 30 s gave similar results (data not shown). Total protein was determined according to the method of Lowry et al. (17) by using different concentrations of bovine serum albumin (Sigma, St. Louis, Mo) in distilled water as a standard. The limit of detection in this assay was 0.1 μg of protein per ml.

For electron microscopy, samples of the bacterial suspensions grown in the presence of three-quarters the MIC of ceftazidime or two-thirds the MIC of ciprofloxacin, and of control cultures, were withdrawn after incubation for 6 h (37°C, 100 rpm) and centrifuged (1,800 × *g* for 10 min). Pellets were fixed in phosphate buffer (0.07 M, containing 2.5% glutaraldehyde [Serva, Heidelberg, Germany] and 0.07% ruthenium red [Merck, Darmstadt, Germany]) overnight at 4°C. A 90-min fixation with phosphate buffer containing 2% osmium tetroxide and 0.07% ruthenium red followed. After being dehydrated in ascending ethanol series, bacteria were embedded in ultra-low-viscosity medium (UVLM) (Polysciences Inc., Warrington, Pa.) twice for 6 h each time and then polymerized at 60°C for 48 h in UVLM and BEEM capsules (Plano, Marburg, Germany).

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Ultrathin sections (70 to 80 nm) were made by using an ultramicrotome (Autocut E; Reichert, Wien, Austria) and examined in a Zeiss EM 10 electron microscope (Zeiss, Oberkochen, Germany). To determine capsular size, exact transverse sections of bacteria were photographed, capsular diameters were measured at four different positions of each bacterium, and the real diameter was calculated from the mean measured diameter.

Levels of CPS were compared on the basis of micrograms of total protein by expressing the ratio of soluble or cell-bound CPS/total protein. CFU counts and amounts of CPS were compared by using the Mann-Whitney U test (16). Diameters of CPS obtained by electron microscopy were compared by using the unpaired *t* test (16). The significance of dose dependence was determined by comparing the values obtained under the influence of the MIC or sub-MIC of the respective antimicrobial agent to the values for the control culture for each time point. The significance of time dependence was determined for each growth condition separately by comparing the first value obtained with the values obtained for the following time points.

RESULTS

MICs of ciprofloxacin and ceftazidime for the test strain were 0.03 and 0.25 μg/ml, respectively. Figure 1 shows the growth curves of *K. pneumoniae* B5055 in the presence of various concentrations (MICs and sub-MICs) of ciprofloxacin (panel A) and ceftazidime (panel B). Whereas ciprofloxacin at 0.03 μg/ml and ceftazidime at 0.25 μg/ml produced growth curves not different from those obtained with the initial CFU transferred, any concentrations less than the MICs resulted in CFU counts hardly significantly different at all from those of the untreated bacteria.

Compared with values for untreated controls, production of soluble CPS as measured by the ratio of soluble CPS/total protein was significantly elevated when antimicrobial agents were added at MICs and sub-MICs (Fig. 2). This effect was time and dose dependent and was achieved with both ciprofloxacin and ceftazidime (Fig. 2A and B, respectively). There was no significant time-dependent change of the ratio in the controls. However, a significant dose-dependent, but not time-dependent, increase in the ratio of cell-bound CPS/total protein could be seen only in the ciprofloxacin-treated samples (Fig. 3A). When cultures were grown under the influence of ceftazidime, no difference between controls and MIC- or sub-MIC-treated bacteria was detected (Fig. 3B).

By electron microscopy, organisms grown in the presence of either antibiotic showed morphologic changes, including development of irregular and filamentous forms, in comparison with untreated control bacteria. At a higher magnification, capsule diameters of those organisms that had been cut in an absolutely transverse direction were measured and were found to be significantly larger in antibiotic-treated than in control bacteria. Control bacteria had a capsule diameter of 29.5 ± 5.3 nm (mean ± standard deviation [SD]) whereas ciprofloxacin-treated organisms showed a capsule diameter of 56.9 ± 17.9 nm (mean ± SD) at 6 h of culture ($P = 0.001$ versus controls by the *t* test). Under the influence of ceftazidime, the bacteria showed a similar enlargement of the capsule diameter (47.9 ± 18.3 nm [mean ± SD; $P = 0.002$ versus controls by the *t* test]). Qualitatively, the capsule of antibiotic-treated bacteria showed a relatively loose structure with occasional clumping of capsular material.

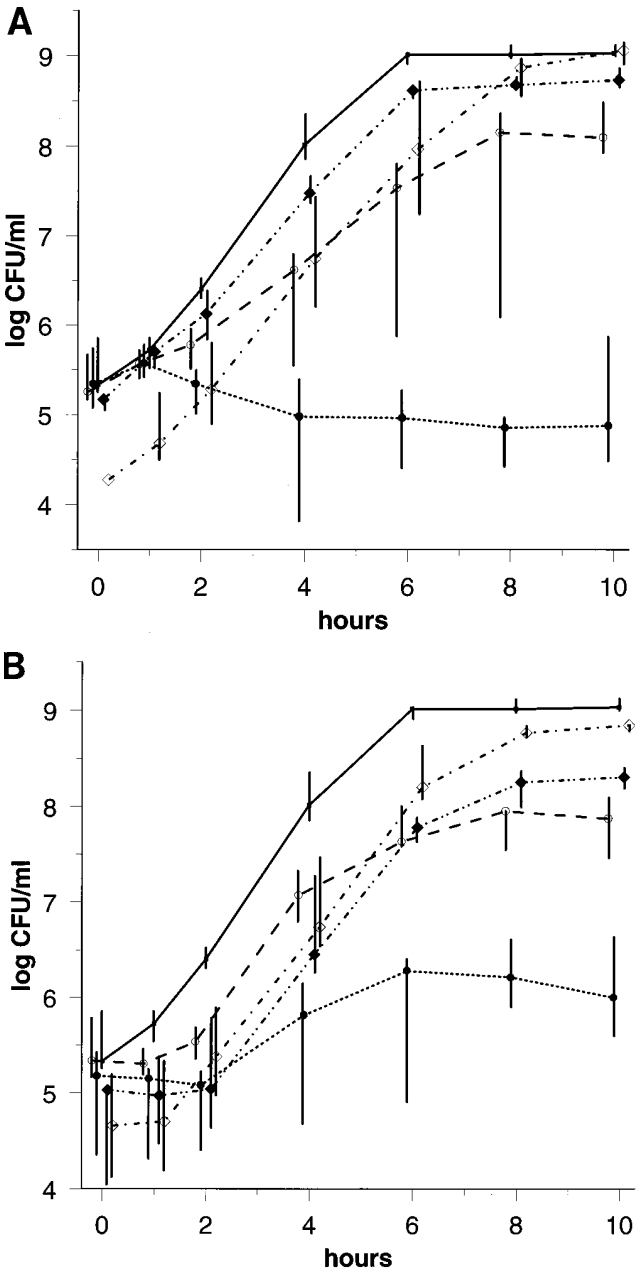

FIG. 1. Growth kinetics of *K. pneumoniae* B5055 obtained with different sub-MICs and MICs of ciprofloxacin (A) and ceftazidime (B). The MIC of ciprofloxacin is 0.03 μg/ml, and that of ceftazidime is 0.25 μg/ml; two-thirds of the MIC of ciprofloxacin is 0.02 μg/ml; three-quarters of the MIC of ceftazidime is 0.1875 μg/ml; one-third of the MIC of ciprofloxacin is 0.01 μg/ml; one-half of the MIC of ceftazidime is 0.125 μg/ml; 1/30 of the MIC of ciprofloxacin is 0.001 μg/ml; one-quarter of the MIC of ceftazidime is 0.6125 μg/ml. Values are given as medians and quartiles. Vertical bars indicate SDs. Lines in panel A: —, control; ···, ciprofloxacin at MIC; - - -, ciprofloxacin at two-thirds the MIC; - · - · - ·, ciprofloxacin at one-third the MIC; - - - -, ciprofloxacin at 1/30 the MIC. Lines in panel B: —, control; ···, ceftazidime at MIC; - - -, ceftazidime at three-quarters the MIC; - · - · - ·, ceftazidime at one-half the MIC; - - - -, ceftazidime at one-quarter the MIC.

DISCUSSION

CPSs, or K antigens, play an important role in bacterial pathogenesis primarily by shielding the microorganisms against the bactericidal activity of complement and engulfment by phagocytic cells (21). Exposure of gram-negative bacilli to sub-MICs of antimicrobial agents such as β-lactams and quinolones has been shown to result in morphological changes such as filamentation (2, 22). In this respect, our results are in accordance with the findings of other authors (8, 22, 25, 27), indicating that, at least in general, similar experimental growth conditions were achieved.

Effects of sub-MICs of various antimicrobial agents on the

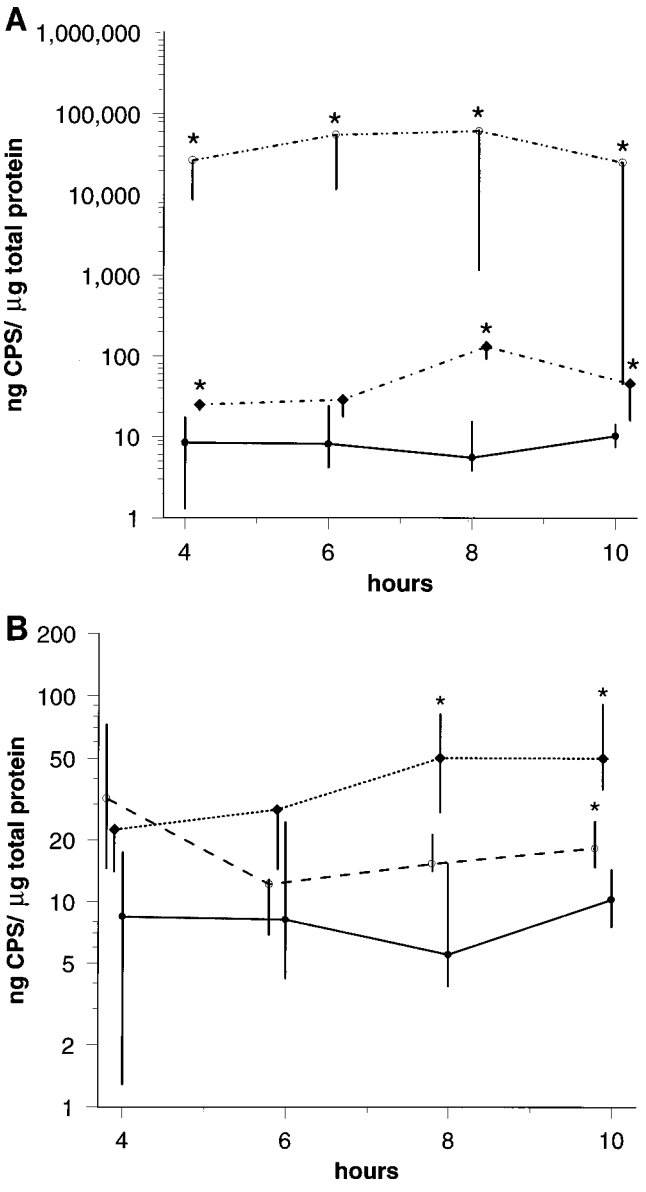

FIG. 2. Ratio of soluble CPS/total protein influenced by sub-MICs and MICs of ciprofloxacin (A) and ceftazidime (B). Each point represents duplicate determinations for three independent experiments. The MIC of ciprofloxacin is 0.03 μg/ml, and that of ceftazidime is 0.25 μg/ml; two-thirds of the MIC of ciprofloxacin is 0.02 μg/ml; three-quarters of the MIC of ceftazidime is 0.1875 μg/ml. Values are given as medians and quartiles. *, $P < 0.05$ versus controls (Mann-Whitney U test). Vertical bars indicate SDs. Lines in panel A: —, control; - - - - - -, ciprofloxacin at MIC; — · — · —, ciprofloxacin at two-thirds the MIC. Lines in panel B: —, control; · · · · ·, ceftazidime at MIC; - - - -, ceftazidime at three-quarters the MIC.

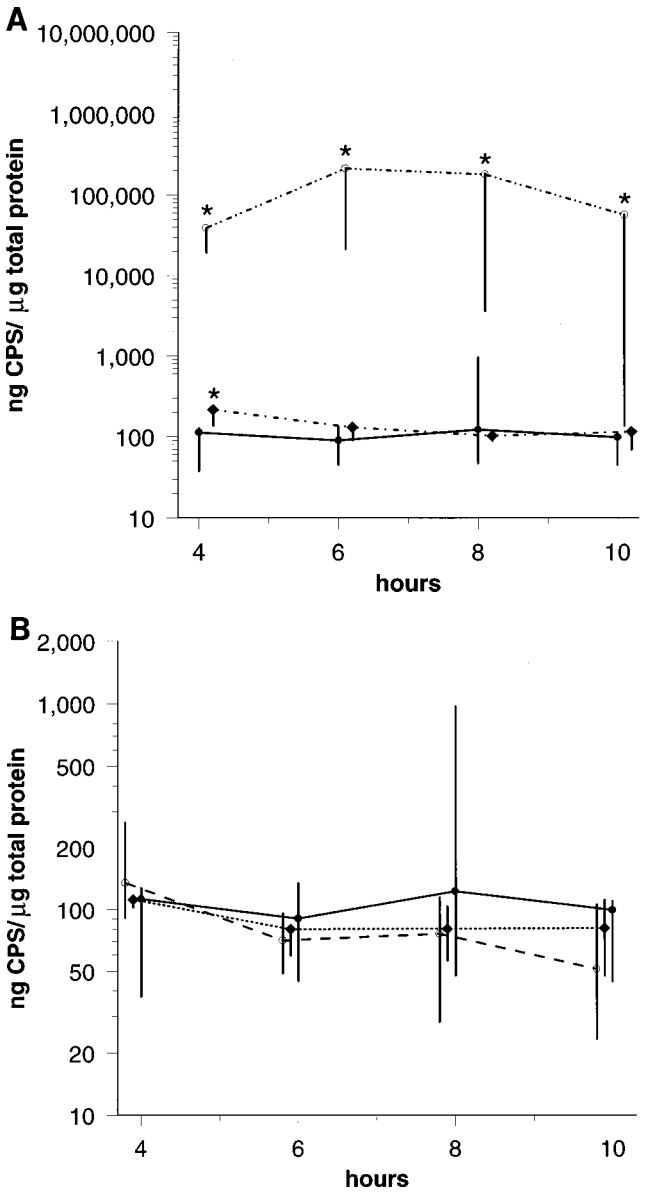

FIG. 3. Ratio of cell-bound CPS/total protein influenced by sub-MICs and MICs of ciprofloxacin (A) and ceftazidime (B). Each point represents duplicate determinations for three independent experiments. The MIC of ciprofloxacin is 0.03 μg/ml, and that of ceftazidime is 0.25 μg/ml; two-thirds the MIC of ciprofloxacin is 0.02 μg/ml; three-quarters the MIC of ceftazidime is 0.1875 μg/ml. Values are given as medians and quartiles. *, $P < 0.05$ versus controls (Mann-Whitney U test). Vertical bars indicate SDs. Lines in panel A: —, control; - - - - - -, ciprofloxacin at MIC; - - - - -, ciprofloxacin at two-thirds the MIC. Lines in panel B: —, control; · · · · ·, ceftazidime at MIC; - - -, ceftazidime at three-quarters the MIC.

production of CPS have also been reported, suggesting a decrease in capsule production (12–14, 27). However, it is important to discriminate between soluble (released) and cell-bound CPS. CPS released by bacteria into the local environment may bind potentially protective antibacterial antibodies, while cell-bound CPS may affect binding of opsonins and uptake by phagocytes (23).

Since quinolones and cephalosporins cause elongation and filamentation of cells (2, 22), we determined the total cell protein at each time point as a parameter of cell mass. Using the ratio of CPS/total protein, we were able to show that under the influence of ciprofloxacin amounts of both soluble and cell-bound CPSs are significantly larger than those in controls and that the effect is dose, but not time, dependent. With ceftazidime, only the level of soluble CPS was significantly elevated above control levels, whereas the ratio of cell-bound CPS/total protein did not differ from that calculated for untreated organisms. We therefore assume that the increase in soluble CPS under the influence of MICs and sub-MICs of both ciprofloxacin and ceftazidime is due to a real increase in

production and does not reflect an increase in cell mass. For cell-bound CPS, an increase in the ratio could be observed only under the influence of ciprofloxacin and not when ceftazidime was used. However, in contrast to previous reports (12, 13), there was no decrease in cell-bound CPS under the influence of ceftazidime.

These differences may be explained in part by the fact that other authors used indirect methods to determine the amount of capsule production, such as surface hydrophobicity and interaction with complement (27), slide agglutination assays with monoclonal antibodies against capsular antigens (14), and polyclonal antisera raised against whole cells of nonencapsulated mutants (12, 27). Since it has been shown that antibodies are able to penetrate the capsule of gram-negative bacteria (15, 26, 28), we think that slide agglutination of encapsulated bacterial strains with antisera or antibodies raised against nonencapsulated strains may not necessarily indicate that the amount of bacterial capsule is decreasing when strains are pretreated with sub-MICs of several antimicrobial agents. It may be that the structure of the bacterial capsule is altered rather than that capsular material is diminished. Our electron microscopy results showed a lower density of the bacterial capsule elaborated under sub-MICs of antimicrobial agents in comparison with the capsule produced without this influence, supporting the former suggestion.

Surface hydrophobicity is believed to increase when CPS is diminished (12, 13, 19). However, it has been shown that neither K^+ O^- nor K^- O^+ mutants of *K. pneumoniae* were distinguishable by two different techniques to measure surface hydrophobicity whereas a K^- O^- mutant was markedly more hydrophobic than the other two strains (29), demonstrating that loss of the CPS alone did not render the organism more hydrophobic (29). Thus, the results obtained by other investigators are not necessarily in contrast to those presented here, since none of the indirect methods most often used to determine changes in CPS production reliably differentiate between quantitative changes and structure alterations of the CPS produced.

In conclusion, our data provide the first direct observation of CPS production of *K. pneumoniae* grown in the presence of MICs and sub-MICs of ceftazidime and ciprofloxacin. We were able to demonstrate that these antimicrobial agents induced an increasing production of soluble as well as cell-bound CPS in a time- and dose-dependent manner. Electron microscopy confirmed our observations by showing a thickened but more loosely associated capsule of antibiotic-treated *K. pneumoniae* compared with controls. Studies of other encapsulated bacteria, with direct measuring of CPS production, are clearly needed to confirm these results. Whether the findings reported here are of clinical significance must be determined by repeating these studies in animal models of infection.

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