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Anomalous Cellular Morphology and Growth Characteristics of *Neisseria meningitidis* in Subminimal Inhibitory Concentrations of Penicillin G

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The effects of subminimal inhibitory concentrations of penicillin G on *Neisseria meningitidis* in the presence and absence of selected stabilizers were examined. Subminimal inhibitory concentrations of penicillin G decreased cell numbers and altered both colonial and ultrastructural morphologies of this meningococcus. Although these levels of penicillin did not have immediate adverse effects on cell mass increase, deoxyribonucleic acid synthesis, or the incorporation of [^{3}H]leucine into protein, they did significantly alter the division rate and the integrity of the cell envelope. The inability of many of the abnormal membranous cell types produced in subminimal inhibitory concentrations of penicillin to form either complete or properly oriented division septa and the overproduction of cell wall material at such sites was indicative of the disruptive effects of this antibiotic on functions necessary for maintaining the normal division process. The addition of the stabilizers polyvinylpyrrolidone-40 and horse serum to the test medium diminished the effects of penicillin G as evidenced by a fourfold increase in the minimal inhibitory concentration. Moreover, such stabilizers maintained the association of the outer membrane with the peptidoglycan and inner membrane.

The exact mechanism(s) by which penicillin compounds exert their bactericidal effect is still not understood. The fact that β-lactam antibiotics can invoke a wide spectrum of morphological and physiological responses in a variety of bacteria has made this problem more complex. Additionally, the action of β-lactam compounds is often not an all-or-none phenomenon, i.e., the antibiotic may also exert effects at concentrations below the minimal inhibitory concentration (MIC) of an antibiotic for a particular organism. Such low concentrations, often referred to as subminimal inhibitory concentrations (sub-MIC's), have been known to affect the morphology of bacteria since the early report on penicillin by Gardner in 1940 (10). After Gardner's work, the production of abnormal bacterial morphology by β-lactam compounds has been reported for a variety of gram-negative organisms (6, 9, 13, 15, 18–21) and gram-positive organisms (6, 17–19). In addition to the phenotypic changes associated with such low levels of antibiotics, changes in growth rates and diminution in the total bacterial population have also been shown in a number of instances (7, 11–13, 16, 24).

The observed effects at sub-MIC levels indicate that β-lactam compounds in general do exert some degree of detrimental and, in a portion of the microbial population, bactericidal activities. Whether these detrimental effects reflect simply a lesser degree of antibacterial action than that which occurs at the MIC or higher concentrations is not understood.

Earlier, we reported (22) that only certain events resulting from low concentrations (approximate MIC) of penicillin G (Pen G) were directly related to a loss of meningococcal viability. Our present study examines the effects of sub-MIC's of Pen G on meningococcal growth and ultrastructure.

MATERIALS AND METHODS

Organism. The smooth colony forms (5) of group B *Neisseria meningitidis* SD1C used in these experiments were obtained from the *Neisseria* Repository, NAMRU, School of Public Health, University of California, Berkeley, and maintained as described elsewhere (3).

Cell growth and Pen G treatment. Growth of *N. meningitidis* SD1C in the presence and absence of supplements was as described by Neirinck et al. (22). Working cultures were incubated for approximately 4 h until they reached an absorbance of approximately 0.6 at 600 nm. At this point, cells were either removed for use in MIC determinations or treated with Pen G (Sigma Chemical Co.) after removal of a sample for analysis (designated as zero time). The Pen G was prepared as previously described (22), and the final concentration of Pen G was 0.016 μg/ml (0.5 MIC).

MIC determinations. The MIC was determined by the agar plate dilution method of Ericsson and Sherris (8).

Electron microscopy. Preparations for electron microscopy were carried out essentially by procedures described by Neirinck (22). Samples on plates were prefixed by flooding the plates with 0.5% glutaraldehyde in 0.15 M sodium phosphate (pH 7.5) and either 0.15 M sucrose or 7% polyvinylpyrrolidone-40 (PVP). Sections were examined with a Philips EM300 electron microscope at 60 kV.

Quantitation of cells. Absorbance (600 nm) and viable counts were measured by the methods previously described (4).

Incorporation of radioactivity into DNA and protein. The syntheses of deoxyribonucleic acid (DNA) and protein were monitored separately by the uptake of [^{3}H]thymidine (21.8 mCi/μmol) and [^{3}H]-leucine (120 mCi/μmol), respectively. Radioisotopes were purchased from New England Nuclear Corp. At 30 min before the addition of Pen G, either [^{3}H]thymidine (1 μCi/ml) or [^{3}H]leucine (5 μCi/ml) was added to the culture. At specified time intervals thereafter, 1.0- and 1.5-ml samples were taken from the [^{3}H]leucine and [^{3}H]thymidine cultures, respectively. These were centrifuged at 12,900 $\times$ g for 2 min in an Eppendorf model 3412 centrifuge. Pellets containing [^{3}H]leucine were suspended in 1.0 ml of cold 5% (wt/vol) trichloroacetic acid and left overnight at 4°C. For measurements of [^{3}H]thymidine incorporation into DNA, cell pellets were first suspended in 0.35 N KOH at 37°C for 17 h. The DNA from these samples was then precipitated with cold trichloroacetic acid (5% final concentration) and collected by filtration on Whatman GF/A glass microfiber filters. The filters were washed five times with 1 ml of cold 5% trichloroacetic acid, air dried, and placed in counting vials containing 10 ml of Aquasol (New England Nuclear Corp.). Radioactivity was measured in a Packard model 3375 liquid scintillation spectrometer.

Incorporation of radioactivity into peptidoglycan. The synthesis of peptidoglycan was measured by the incorporation of [^{3}H]glucosamine (19 mCi/μmol; New England Nuclear Corp.) into the fraction insoluble in sodium dodecyl sulfate. At 15 min before the addition of Pen G, [^{3}H]glucosamine (2 μCi/ml) was added to the culture. At specified time intervals thereafter, 1.5-ml samples were removed and processed for the isolation of peptidoglycan by using a modified procedure of Hebler and Young (14). Samples were added directly to tubes containing 8 ml of 4.75% (wt/vol) sodium dodecyl sulfate (Sigma Chemical Co.) at 100°C and 0.3 ml of unlabeled carrier peptidoglycan (5 mg/ml) previously isolated as described here. All samples were incubated for 4 h at 100°C. The sodium dodecyl sulfate-insoluble peptidoglycan was subsequently collected by centrifugation at 100,000 $\times$ g for 45 min at 20°C in a Beckman L5-65B ultracentrifuge with a 60 Ti rotor. After the peptidoglycan was washed twice with 20 ml of distilled water, the final pellet was suspended in 1.0 ml of distilled water. Duplicate 0.3-ml samples of each were placed in counting vials together with 10 ml of Aquasol, and radioactivity was determined as described above.

RESULTS

A recent report from this laboratory (22) had shown that the MIC of Pen G for *N. meningitidis* SD1C is 30 ng of Pen G per ml. No "inoculum effect" or production of mutants could be obtained even when the concentration of the inoculum was increased from 1×10^6 to 1.5×10^9 colony-forming units. Others (23) have reported that an increase in the size of the inoculum during susceptibility testing is sometimes accompanied by the appearance of resistant cells in meningococcal populations.

At sub-MIC's of Pen G, the cells in the population in our experiments were not uniformly affected by this antibiotic. Quantitation of the surviving cells at sub-MIC's revealed that only 10 to 15% of cells survived at 0.5 MIC (15 ng of Pen G per ml), whereas 100% of the cells survived at 0.125 MIC (4.0 ng of Pen G per ml). The differential susceptibility in the meningococcal population to the presence of 0.5 MIC of Pen G was manifested also by a marked change in the colonial size and morphology produced by surviving cells. Furthermore, the subculture of any one of the colonies onto plates with the same medium and Pen G concentration produced a similar variety of colonial sizes and morphologies, indicating that genetic mutation to resistant forms was an unlikely explanation for such variation. All variant colonies, when subcultured onto Mueller-Hinton agar without Pen G, readily produced the colonial morphology and size typical for normal growth in the absence of Pen G.

The finding that only 10 to 15% of the cells in the population were able to survive at 0.5 MIC suggests that such cells have a selective advantage (other than genetic resistance) for survival. It is possible that certain cells in a population of genetically identical cells may have expressed different phenotypes, which could account for the survival of a small proportion of cells even though they had been adversely affected by Pen G. Isolated membranes and aberrant cell forms were observed in all populations, but there was always present a small number of apparently normal cells. The proportion of these normal cells was dependent upon the concentration of the Pen G used. In Fig. 1, the spectrum of effects due to a 0.5 MIC of Pen G during cell growth is shown. The surviving intact cells exhibited irregular division patterns, variations in size, and a loss of cell envelope integrity. Moreover, many of the cells overproduced cell wall material, which appeared to be peptidoglycan, in the zone of the newly formed division septum (Fig. 1). Noteworthy was the apparent cellular division

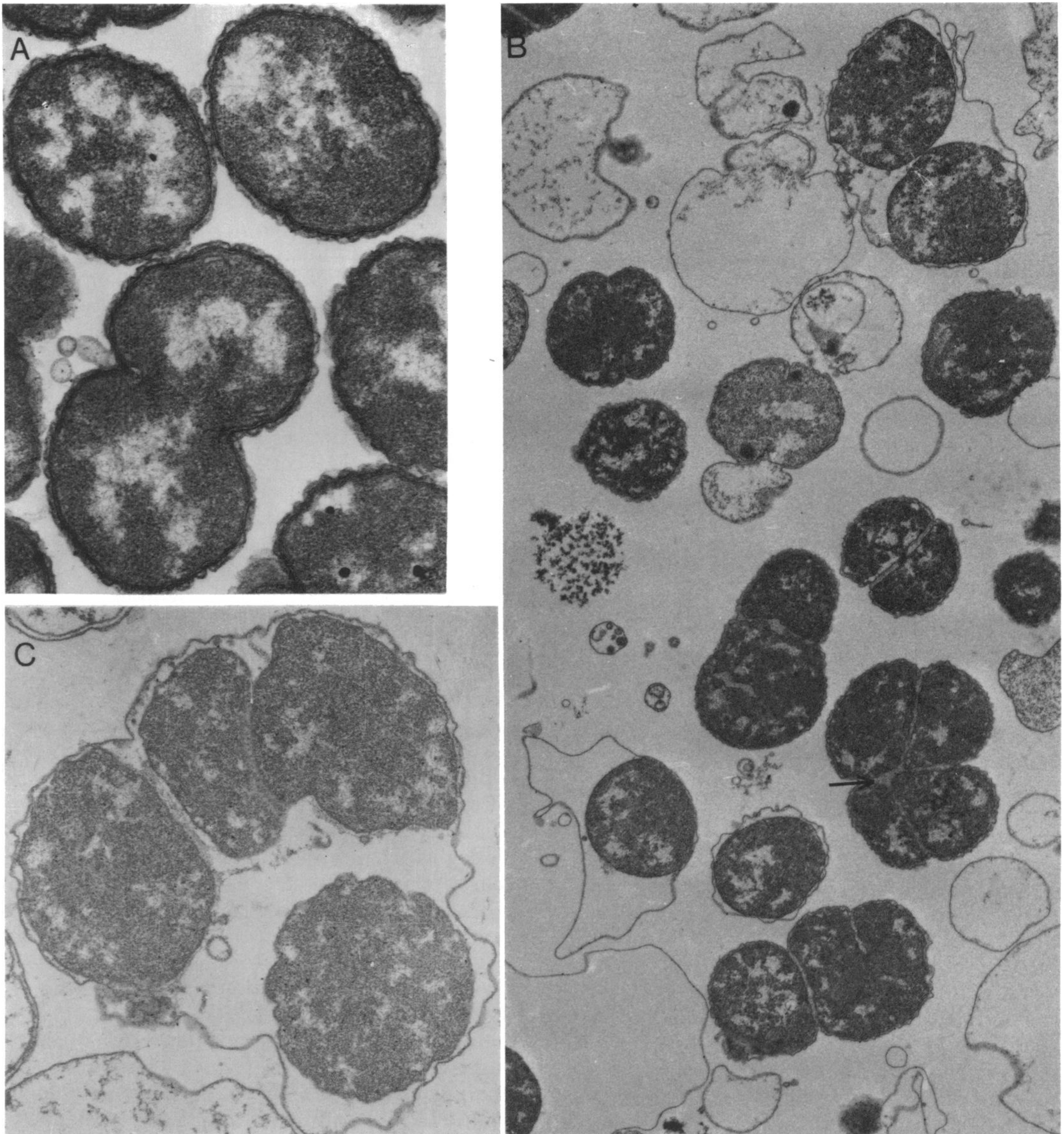

FIG. 1. *Thin sections of N. meningitidis cells in (A) absence of Pen G (×45,000) and (B) presence of Pen G (16 ng/ml). The sample in (B) shows abnormal cellular morphologies and division processes. The arrow indicates a site of overproduction of cell wall material (×13,300). (C) The division of cell protoplasts in the absence of outer membrane involvement (×30,000).*

of the cell protoplast in the absence of outer membrane involvement (Fig. 1C), an event also observed when Pen G was used at concentrations above the MIC (22).

Since cells in broth cultures displayed the same dramatic morphological changes as those on solid medium at sub-MIC's, the broth medium was used to study selected growth characteristics. The results indicate that although there was little or no change in absorbance of the culture (data not shown) until 120 min after the addition of 0.5 MIC of Pen G, there was an immediate effect on the rate of cell division (Fig. 2). At this reduced concentration of Pen G, the viability fell off slowly after approximately one doubling of the population, until after 4 h only 15 to 20% of the cells were capable of forming colonies in the absence of Pen G. This low death

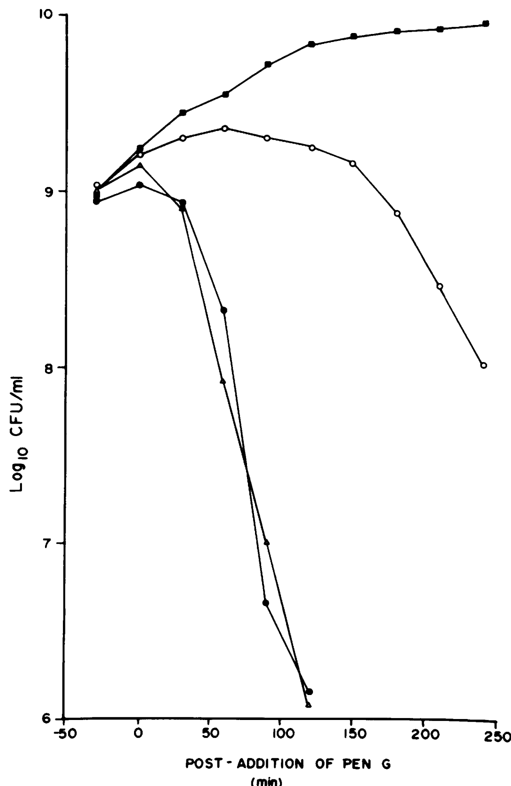

FIG. 2. *Viability changes after the addition of Pen G to a rapidly growing broth culture of N. meningitidis. Symbols: ■, without Pen G; △, with Pen G, 2.0 μg/ml; ●, with Pen G, 40 ng/ml; ○, with Pen G, 16 ng/ml.*

rate indicates that cell death in the population below the MIC must be of the first order with respect to the Pen G concentration and is unlike the rapid logarithmic death in populations in Pen G at concentrations higher than the MIC, previously shown to be a zero-order reaction (22). All data taken together suggest that at the 0.5 MIC of Pen G it is the cell division process itself which is disrupted. The progressive increase in turbidity of the culture without a simultaneous increase in the number of colony-forming units was the result of the progressive cellular mass increase. Such cells were incapable of normal cell division but resisted lysis over the course of the experiment. The continued growth until colonies are visible on solid medium may well involve the same abnormal cell division process.

We had previously shown (22) that, even in the presence of Pen G slightly above the MIC, macromolecular synthesis continued for up to 90 min. Results (data not shown) indicate that the rates of synthesis of DNA and protein, as measured by the incorporation of [^{3}H]thymidine and [^{3}H]leucine, respectively, continued to parallel those of the control cells for at least 180 min after the addition of Pen G. In addition, the incorporation of [^{3}H]glucosamine into insoluble peptidoglycan showed that the production of the sodium dodecyl sulfate-insoluble polymer was not significantly affected for at least the first 60 min after addition of Pen G. Although hydrolysis of insoluble peptidoglycan was not measured, the small difference in net incorporation of glucosamine that was observed in the presence of Pen G may represent a mild stimulation of hydrolytic activity rather than an inhibition of transpeptidation. The results of glucosamine incorporation must be interpreted in light of the heterogeneous changes observed in the cell population in a 0.5 MIC of Pen G.

We had previously shown (22) that the addition of the stabilizing components PVP and horse serum to broth cultures with Pen G at concentrations above the MIC protected meningococci and raised the MIC significantly from 30 to 125 ng of Pen G per ml. The PVP plus horse serum in the inoculum could not account for the effects observed. When cells were plated onto a medium containing 30 ng of Pen G per ml (MIC), a concentration which does not permit cell growth, colonies were formed with the addition of the 40,000-molecular-weight polymer PVP plus horse serum. Again, cell growth was characterized by altered colonial sizes and morphologies. In the electron microscope, cells in thin section exhibited abnormalities related to cell division, e.g., abnormal planes of division and relative overproduction of cell wall material (Fig. 3). However, unlike organisms grown at the sub-MIC of Pen G without PVP plus horse serum, the envelope integrity in these cells was maintained, and there was no indication of aberrant membranous cell forms. The numerous small membranous blebs released from the meningococcal outer membrane have previously been shown by DeVoe and Gilchrist (4) to be a normal consequence of cell growth. The results suggest that the continued presence of PVP plus horse serum during exposure to Pen G not only maintains a higher degree of viability in the population but acts to eliminate those changes in the cell envelope normally associated with the actions of Pen G.

DISCUSSION

Previous work on the action of β-lactam antibiotics on bacterial populations indicates that their action is not all or none (11, 12, 24). Among the effects of sub-MIC's on bacteria are the significant morphological modifications and

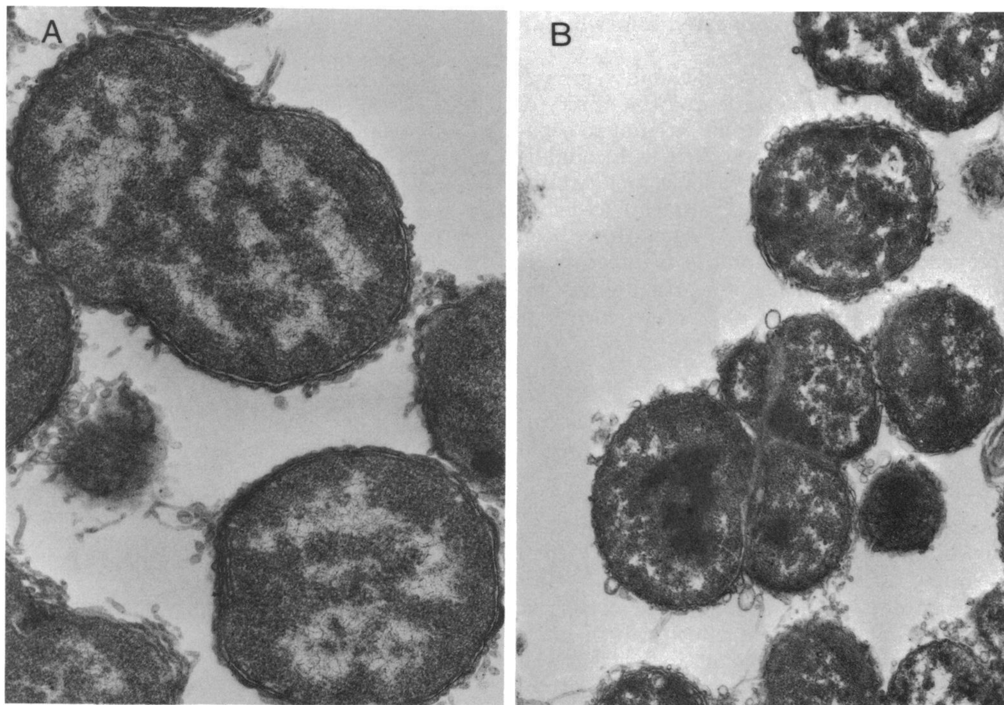

FIG. 3. *Thin sections of N. meningitidis growing on solid medium supplemented with PVP and horse serum. (A) Without Pen G (×38,400); (B) with 30 ng of Pen G per ml (nonpermissive in the absence of stabilizers) (×19,800).*

physiological alterations in the cell division cycle and a reduction in the number of viable cells in the population. Our work presented here describes effects on *N. meningitidis* exposed to a sub-MIC of Pen G. At sub-MIC's, there is a differential susceptibility among the cells in the population, which is unrelated to inoculum size or to genetic variation to more resistant cell types. Such variation may well reflect subtle phenotypic differences between cells. The altered colonial morphology, variable colony size, and slow growth rate of the survivors are all manifestations of an altered cellular growth process within the colonies. The presence of Pen G, even after dilution to 0.125 MIC (4 ng of Pen G per ml), continued to cause abnormal colonies despite the fact that there was no loss in viability.

Previous reports by Lorian and Atkinson (18, 19) on the action of sub-MIC's of Pen G on gonococcal ultrastructures show that cells may increase slightly in size and have increased cross wall thickness, but exhibit normal peripheral cell wall structures. Examination by electron microscopy of the response of *N. meningitidis* to sub-MIC's of Pen G in broth and solid media revealed a more severe response than that of *Neisseria gonorrhoeae*. Overproduction of cell wall material in the septal region, appearing as peptidoglycan, was frequently observed, but it was not associated with the more severely affected cells. With time, this excess cell wall material at the septum and the entire peptidoglycan surrounding the cell was completely lost with a sub-MIC of Pen G. This suggests that the sizeable accumulation of peptidoglycan material at the septum was a temporal event in the final outcome of Pen G action at these low concentrations. The two most striking ultrastructural changes with Pen G sub-MIC's were the abnormal orientation in planes of septum formation and the loss of outer membrane attachment to the peptidoglycan and inner membrane. These two events together correlated with the inability of these cells to divide. The effect of a sub-MIC of Pen G on growth rates was immediate, yet a decrease in viability did not start for over 60 min after the addition of Pen G. Despite the altered growth rate, other characteristics, i.e., absorbance, protein synthesis, and DNA replication, suggested that the cells were otherwise normal. By all criteria tested, most cells were metabolically active but were incapable of continuing the cell division process for prolonged periods and, therefore, eventually lost viability with time in the presence of Pen G. Only the production of

insoluble peptidoglycan appeared somewhat altered after 60 min in Pen G.

Sub-MIC's of Pen G affected viability, growth rates, cell envelope integrity, and the ability of cells to divide normally. In an attempt to separate the lethal events from those which could be considered nonlethal or reversible, the stabilizing agents PVP and horse serum were added to the medium with Pen G. These agents have been employed by others to stabilize meningococcal L-forms (2). PVP has also been used in susceptibility testing of certain antibiotics since it is thought to represent more closely an in vivo environmental condition (1). When *N. meningitidis* was tested with Pen G and PVP plus horse serum in the medium, there was an increase in MIC (fourfold), but the atypical colonial morphology and slow growth rate on the solid medium did not change. These results indicate that PVP and horse serum play a role in maintaining viability by methods other than prevention of altered morphology and slow growth. After a closer examination of cells at Pen G concentrations that allow growth, PVP and horse serum were found to prevent the detachment of outer membrane from peptidoglycan from PG and inner membrane, i.e., the stabilizers prevented the production of the aberrant membranous forms. Since this is the only observable difference in the presence of the stabilizers, maintaining correct outer membrane integrity appears important in preventing cell death. This correlates well with results from an earlier report (22) on meningococcal responses to concentrations equal to or higher than the MIC of Pen G in the presence and absence of PVP and horse serum. The mechanism by which the outer membrane is lost from cells and the exact implications of such loss in the killing action of Pen G remain matters for speculation.

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