Penicillin-Resistant and Penicillin-Tolerant Mutants of Group A Streptococci

LAURENT GUTMANN AND ALEXANDER TOMAZS*

The Rockefeller University, New York, New York 10021

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Penicillin-resistant and penicillin-tolerant mutants have been isolated from group A streptococci mutagenized by ethyl methane sulfonate. The resistant mutants had an elevated minimal growth inhibition concentration for benzylpenicillin (minimal inhibitory concentration, 0.2 μg/ml, as compared with the minimal inhibitory concentration of 0.006 μg/ml in the penicillin-susceptible parent strain); they also had an abnormal cellular morphology and showed altered penicillin-binding proteins. Penicillin-tolerant mutants were killed more slowly than were the parental cells during treatment with penicillin; they had virtually unchanged minimal inhibitory concentration values for penicillin and normal cellular morphology and penicillin-binding proteins.

Group A streptococci are causative agents of a variety of human diseases, and penicillin has been widely used in clinical medicine as a chemotherapy of choice. This antibiotic is known to cause rapid loss of viability in growing cultures of group A streptococci without apparent lysis of the cells (12). Although resistant mutants have been isolated in the laboratory, there has been no report of a shift toward a higher level of penicillin resistance in natural isolates, despite the extensive use of penicillin for prophylaxis in acute and chronic forms of the disease. Penicillin resistance has never been observed during the routine testing of the antibiotic susceptibilities of group A streptococci isolated in cases of treatment failures (R. Facklam, Centers for Disease Control, Atlanta, Ga., personal communication). In contrast, the emergence of penicillin-resistant forms has been frequently reported among α-hemolytic streptococci of the oral flora of patients on prolonged penicillin therapy (20).

Penicillin-tolerant isolates, i.e., group A streptococci with increased penicillin minimal bactericidal concentration values relative to the penicillin minimal inhibitory concentration (MIC) values have been observed in clinical specimens (1).

Up to the late 1960s, resistance to non-beta-lactam antibiotics was rare in group A streptococci. Since the early 1970s, however, large increases in the frequency of resistance of clinical isolates to sulfonamides, tetracycline, chloramphenicol, and erythromycin have been reported, and several hospital surveys have indicated that such resistant bacteria may make up a substantial fraction of current clinical isolates. In addition, mechanisms for the cell-to-cell transfer of resistance genes have also been described (13, 24). The emergence of resistance in group A streptococci is reminiscent of the situation described for pneumococci in the late 1970s, when multiply antibiotic-resistant organisms carrying high levels of penicillin resistance suddenly appeared in hospital isolates in several locations (14). These penicillin-resistant pneumococcal strains were shown to contain altered penicillin-binding proteins (PBPs) (25).

In the present communication, we describe the laboratory isolation and preliminary characterization of penicillin-resistant and penicillin-tolerant group A streptococcal mutants. It is hoped that future studies with these mutants will provide useful information about the mechanism of action of penicillin in these bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Streptococcus pyogenes T4/56 (obtained from M. McCarty, The Rockefeller University, New York, N.Y.) was grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.) in 10-ml batch cultures in 18-mm culture tubes at 37°C in air without shaking. Growth was monitored with a Coleman nephelometer.

Isolation of penicillin-resistant mutants. An exponentially growing culture of S. pyogenes T4/56 in THB at a cell concentration of about 7 × 10^9 colony-forming units (CFU) per ml was treated with ethyl methane sulfonate (150 μl/10 ml of culture) at 37°C for 2 h (16). This treatment caused the loss of about 90% of the CFU. After removal of the mutagen, the surviving bacteria were grown in THB, and 0.1-ml portions of this culture (containing about 8 × 10^6 CFU) were used to inoculate test tubes containing 10 ml of THB and a series of concentrations of penicillin ranging from 0.003 to 0.2 μg/ml. The cultures were incubated at 37°C for 48 h; 0.1 ml of bacteria from the tube with the
highest concentration of penicillin which permitted growth was used as an inoculum for a second set of culture tubes containing penicillin, as described above (17). Bacteria growing in the medium with 0.2 μg of penicillin per ml were plated on brain heart infusion agar plates containing 5% blood and penicillin (0.1 μg/ml). Resistant mutants were purified by two rounds of single-colony isolation. Stocks were stored in THB medium with 10% glycerol at −70°C. A single mutant isolate, P23, was used for detailed study.

**Stability of the penicillin-resistant mutant P23.** Five colonies (P423, P623, P723, P923, and P1023) derived from the resistant mutant P23 were subcultured 10 times (100-fold dilution each time) in penicillin-free medium, and the plating efficiency of the isolates was compared on penicillin-free and penicillin-containing agar plates. The test was done in the following way. One hundred to 300 CFU were plated on penicillin-free blood agar master plates. After 18 h at 37°C, a replica plating was done onto a series of blood agar plates containing increasing concentrations of penicillin. At 0.1 μg of penicillin per ml, which was the lowest concentration at which P23 was still able to grow, the plating efficiency was 90 to 100%, except for P923, which had a low plating efficiency (about 2%) at this concentration of penicillin but was fully able to grow on plates with 0.05 μg of penicillin per ml.

**Tolerant mutants.** Tolerant mutants were obtained from a mutagenized culture as described above. A 0.1-ml amount of the mutagenized culture was inoculated into 10 ml of THB and incubated until the optical density reached 200 nephelometric units (approximately 5 × 10^6 cells per ml), at which time 0.1 μg of penicillin per ml was added (about 15 times the MIC). After 6 h of incubation, the cells were washed two times with sterile THB (centrifugation) and suspended in 10 ml of antibiotic-free THB. One milliliter of this suspension was inoculated into 9 ml of THB and incubated overnight. The penicillin treatment (0.1 μg/ml for 6 h) was then repeated six times to allow the enrichment of penicillin-tolerant cells. Finally, 0.1 ml of such a culture (approximately 5 × 10^6 CFU) was spread onto blood agar plates containing 0.2 μg of penicillin per ml and incubated for 12 h at 37°C. Surviving bacteria were replated with sterile velvet onto blood agar plates without penicillin and incubated at 37°C for 40 h. About 40 colonies were isolated on each plate by this method. Each clone used in this work was retested for the homogeneity of its tolerant response to penicillin in the following manner. About 100 to 300 CFU were plated on blood agar master plates, and after 12 h of incubation at 37°C, the colonies were replica plated onto blood agar plates containing 0.2 μg of penicillin per ml. These latter plates were incubated at 37°C for 12 h, at which time a second replica plating was done onto blood agar plates free of penicillin. Virtually all of the colonies survived this penicillin challenge, as evidenced by the recovery of 95 to 100% of the colonies of the master plate. The same treatment resulted in the loss of 100% of the parental wild-type streptococci (no survivors among 200 to 300 colonies). Two tolerant mutants, Tol23 and Tol57, were chosen for detailed study.

**Testing of antibiotic susceptibility.** The MIC was determined by the tube dilution method (21) in THB. With an inoculum of 10^4 to 10^5 CFU/ml and an incubation time of 20 h, the viable titer of cultures was determined by the standard procedure for counting colonies on blood agar plates. Variation in the inoculum size (between 10^3 and 10^5 CFU/ml) did not change the MIC values. Organisms were diluted in saline; 0.1-ml volumes of each sample were spread onto plates and incubated for 36 h at 37°C.

**Osmotic protection of penicillin-treated cells.** Bacteria grown and treated with penicillin in THB were plated on blood agar plates supplemented with hypertonic sucrose and sodium chloride (brain heart infusion broth; 1.2% agar–0.6 M sucrose–0.25 M NaCl). Alternatively, the bacteria were grown and treated with penicillin in liquid THB medium supplemented with sucrose and sodium chloride at the above concentrations and then plated on agar with or without osmotic supplements.

**Leakage of prelabeled protein and RNA from group A streptococci.** An exponential-phase culture of bacteria in THB supplemented with uracil (5 μg/ml) was divided into two 12-ml samples which received either 3 μCi of [3H]uracil or 18 μCi of [35S]methionine per ml. Further incubation at 37°C was continued for four generations. The cells were harvested (12,000 × g, 5 min) and washed three times with cold THB. Each sample was suspended in 25 ml of THB and allowed to grow for 30 min, at which time half of the culture samples received penicillin (0.15 μg/ml). Further incubation was done at 37°C. At intervals, 500-μl samples were centrifuged at 12,000 × g for 10 min. Radioactive material released into the supernatant was assayed in the following way. Two hundred fifty microliters of the supernatant received 2 ml of cold 10% trichloroacetic acid and 25 μl of 4% albumin solution (Armour fraction V). The precipitates were collected on glass fiber (GFA) filters and radioactively determined in a mark II

### Table 1. MIC values for various antibiotics in the penicillin-sensitive (wild-type), penicillin-resistant (P23), and penicillin-tolerant (Tol23) strains of group A streptococci

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (μg/ml) of following strain:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.006</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.03</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.007</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1</td>
</tr>
<tr>
<td>Cephalaridone</td>
<td>0.007</td>
</tr>
<tr>
<td>Cefsulodin</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.015</td>
</tr>
<tr>
<td>Thienamycin (MK 0787)</td>
<td>0.0035</td>
</tr>
<tr>
<td>Sch 29482</td>
<td>0.03</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>180</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>2</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
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</tr>
<tr>
<td>Erythromycin</td>
<td>0.015</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.06</td>
</tr>
<tr>
<td>Aerosporin</td>
<td>16</td>
</tr>
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a In another penicillin-tolerant mutant (Tol57), the MIC for penicillin was 0.01 μg/ml.  

b ND, Not determined.
FIG. 1. Inhibition of growth of wild-type and penicillin-resistant streptococci by penicillin. Exponentially growing cultures of wild-type (WT) and penicillin-resistant (P23) cells were exposed to several concentrations of penicillin (added at the time indicated by the arrows) ranging from 0.01 to 2.0 μg/ml (see numbers on graphs). Control cultures (C) received no antibiotic.

spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.).

In vivo labeling of PBPs. One-milliliter samples of exponentially growing bacteria (about $5 \times 10^7$ CFU/ml) were diluted to identical optical density values and exposed to a series of concentrations of $[3^H]$penicillin for 10 min at 37°C. Nonradioactive penicillin (500 μg) was added, and the bacteria were recovered by centrifugation (12,000 × g at 4°C for 5 min). The cells were suspended in 40 μl of lysis buffer (50 mM sodium phosphate buffer [pH 6.1] containing 5 mM EDTA) in the presence of an excess of nonradioactive penicillin. A lytic enzyme (C-phage associated lysis) prepared by the method of Fischetti et al. (6) was added, and the samples were incubated at 37°C for 10 min. Wild-type and mutant cultures appeared to undergo complete lysis under these conditions, as judged by both the complete clearing of suspensions and the pattern of protein bands visualized by Coomassie blue staining. After the addition of a 25-μl sample of dilution buffer (3), the samples were boiled for 2 min and applied to polyacrylamide slab gels. Polyacrylamide slab gel

FIG. 2. Electron micrographs of the penicillin-sensitive (wild-type) and penicillin-resistant (P23) streptococci. Scanning electron micrographs of the wild-type (A) and mutant (B) cells and thin section of the mutant bacteria (C) are shown. Bars: (A) and (B), 2 μm; (C), 0.5 μm.
RESULTS

The penicillin-resistant bacterium had increased MIC values for a number of structurally diverse beta-lactam antibiotics (Table 1). However, the degree of change in the MIC values varied greatly from one beta-lactam to another. For instance, whereas the MIC values for benzylpenicillin and oxacillin increased about 30-fold, there was relatively little increase (two- to fourfold) in the MIC values for cefoxitin or cephaloridine and none for cefsulodin (Table 1). Susceptibilities to several non-beta-lactam antibiotics remained virtually unchanged. There was a somewhat increased susceptibility to aminoglycosides in mutant P23.

Penicillin-resistant mutants. Inhibition of growth of the penicillin-resistant mutant P23 (benzylpenicillin MIC, 0.2 μg/ml) required substantially higher concentrations of penicillin than did inhibition of the parental penicillin-susceptible strain T4/56 (benzylpenicillin MIC, 0.006 μg/ml) (Fig. 1). Strain P23 also grew more slowly than strain T4/56 in THB (average doubling times, 108 min for P23 and 35 min for T4/56). Addition of penicillin resulted in rapid loss of viability in both strains (data not shown), although the bactericidal effect of penicillin was somewhat slower in P23, perhaps owing to the slower growth rate.

Cells of strain P23 grown in THB had an aberrant morphology (Fig. 2A and B). The abnormal morphology of several subclones was shown to be retained during at least 10 subcultures (involving about 100 cell generations) in penicillin-free THB, and such cultures had the same plating efficiencies on drug-free and on penicillin-containing (0.1 μg/ml) blood agar plates.

Exponentially growing cultures of T4/56 and P23 were briefly exposed to various concentrations of radioactive penicillin, and the labeling patterns of the PBPs were compared (Fig. 3). Quantitative scanning of the labeled PBPs revealed several alterations in PBP 2 of the resistant mutant, suggesting both lower cellular concentration of labeled penicillin and altered labeling patterns.
centration and lower penicillin affinity. The resistant bacteria also appeared to have an altered PBP 3; this protein seemed to have lower antibiotic affinity and was present at a higher concentration than in the susceptible bacteria (Fig. 3 and 4).

**Penicillin-tolerant mutants of group A streptococci.** Several attempts were made to detect cell structural defects in group A streptococci killed by penicillin. Penicillin treatment that caused loss of over 99% of the CFU caused no detectable loss of protein or nucleic acid material from bacteria (loss of less than 1% of the total incorporated label precipitable by cold trichloroacetic acid) prelabeled with the appropriate radioactive isotopes. In addition, an examination of electron microscopic sections of penicillin-treated bacteria revealed no clear sign of structural damage (Fig. 5). On the other hand, a parallel assay of viability of penicillin-treated bacteria on normal blood agar plates and blood agar plates supplemented by high concentrations of sucrose and sodium chloride indicated that a large portion of the cells could be salvaged as viable colony formers on the osmotically supplemented plates (Fig. 6). This finding suggested that, similar to other species of bacteria, group A streptococci may lose viability by a process involving structural damage to the cell wall. If this were the case, then it might be possible to find mutants of group A streptococci specifically blocked in the process(es) involved with the viability loss. We succeeded in isolating such penicillin-tolerant mutants.

Figure 7 shows a comparison of the responses of the parental and two mutant cultures (Tol23 and Tol57) to penicillin treatment. All three cultures grew with comparable rates and showed inhibition of growth by penicillin at the same concentrations and by comparable kinetics. The tolerant mutants had normal morphology. On the other hand, penicillin-induced loss of viabi-
PENICILLIN-RESISTANT GROUP A STREPTOCOCCI

FIG. 5. Morphology of penicillin-treated and control group A streptococci. Bacteria grown in THB were treated with penicillin (20× MIC) for 2 h as in the experiment described in Fig. 1. Control (A) and treated (B) cells were fixed with 2% glutaraldehyde added to the growth media. After a second fixation in OsO₄ (0.5%) and staining with uranyl acetate, the cells were put through a routine cytological procedure (19, 23).

TY occurred only at greatly reduced rates in the tolerant cultures.

The tolerance of mutant cultures was not due to the presence of a subpopulation of slow-growing (and, thus, phenotypically tolerant) cells, since after exposure of several hundred tolerant cells on agar plates containing penicillin (0.2 μg of penicillin per ml for 12 h), all bacteria could be rescued as colony formers after flooding the plates with penicillinase and a subsequent overnight incubation. No viable cells could be recovered from similarly treated wild-type (T4/56) cells. Table 1 shows that the tolerant mutants and wild-type streptococci had virtually identical MIC values for beta-lactam antibiotics. However, a modest increase in the MIC values of several aminoglycosides was noted. A preliminary examination of the PBPs of

FIG. 6. Osmotic protection of group A streptococci treated with penicillin. (A) An exponential culture of wild-type group A streptococci (T4/56) was divided into two 12-ml samples. One received 0.12 μg of penicillin (PEN), and the other served as a control (C). During further incubation at 37°C, samples were taken and, after appropriate dilutions, were spread on blood agar plates (bap) and osmotic plates (osm) (see text) at the same time. (B) An exponential culture of wild-type strain T4/56 was grown in liquid osmotic medium and divided into two 12-ml samples. One served as a control (C), and one received 0.12 μg of penicillin (PEN) per ml (see text). Further incubation was done at 37°C. Samples were taken at intervals, diluted in osmotic medium, and spread at the same time on blood agar plates (bap) and osmotic plates (osm).
tolerant mutants showed a normal complement of PBPs, with a somewhat lower concentration in one of the minor PBPs (PBP 3) (Fig. 3).

DISCUSSION

Although penicillin-resistant group A streptococci have not been reported among clinical isolates, mutants exhibiting elevated MIC values for penicillin have been constructed in the laboratory (5, 17). Some of these resistant isolates were shown to have a lower capacity for the cellular binding of radioactively labeled penicillin (5). The present report, in essence, confirms and extends these earlier observations: in penicillin-resistant mutants, PBPs 2 and 3, proteins responsible for the binding of over 60% of penicillin, were shown to have a decreased affinity for penicillin, and PBP 2 may also be present in lower cellular concentrations. Similar types of PBP alterations have been observed in both laboratory and clinical isolates of several other species of beta-lactam-resistant bacteria, and such PBP alterations may indeed be generally associated with intrinsic beta-lactam resistance (2-4, 7, 9, 10, 25). It is not known how many mutational events are involved with the penicillin-resistant and -tolerant phenotypes of the group A streptococci described in this report.

The relative ease with which penicillin-resistant group A streptococci may be isolated in the laboratory makes one wonder why such bacteria have not appeared in natural isolates despite the extensive use of penicillin at low concentrations in various forms of group A streptococcal disease. One possible cause of this may be a selection against such bacteria in their natural environments, since an apparent decrease in the production of antiphagocytic M-protein has already been observed in the penicillin-resistant mutants of group A streptococci (18). Since PBPs are, presumably, enzymes involved with
the biosynthesis of bacterial cell wall, penicillin-resistant streptococci with their abnormal PBPs may produce sufficiently altered cell walls to hinder the synthesis or attachment or both of nonmurein wall polymers (M-protein) as well. The abnormal morphology of the penicillin-resistant mutants described here is consistent with this suggestion.

Another phenomenon that may also contribute to the relative rarity (perhaps absence) of penicillin resistance among natural isolates of group A streptococci is the cooperative bacterial effect of penicillins and leukocytes that has been observed during exposure of several bacterial species to sub-MICs of penicillin. Studies are in progress to test these possibilities. The nature of the process that is altered in the tolerant mutants and that appears to be the rate-limiting step in the loss of viability of penicillin-treated wild-type cells is not known at the present time. Penicillin treatment of group A streptococci stimulates the release of lipoteichoic acids and lipids into the surrounding medium (11). However, this appears to involve an active process rather than a correlate of viability loss, since streptococci tolerant to penicillin also exhibit penicillin-induced release of cell surface components (12). In other bacteria, penicillin-induced lysis and killing can be correlated with the triggering of murein hydrolase activity and murein degradation (for review, see reference 22). It is conceivable that in group A streptococci also, the loss of bacterial viability involves triggering of a type of murein hydrolase activity that would introduce limited but irreversible nicks into the cell walls without causing complete collapse of cell structure. The observed rescue of penicillin-treated cells from loss of viability by plating on osmotically supplemented agar is consistent with such a mechanism. As an alternative, it has been suggested that in these bacteria, penicillin treatment may cause a membrane level defect, e.g., by activation of phospholipases (22).

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LITERATURE CITED
