Mechanism of Penicillin Killing in the Absence of Bacterial Lysis

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Exposure of group A streptococci (a nonlytic-death phenotype) to benzylpenicillin (penicillin G) produced a dose-dependent, rapid, and extensive hydrolysis of total cellular RNA, with the subsequent loss of hydrolysis products from the cell. This loss of RNA correlated well with loss of viability and was not accompanied by solubilization of the cell wall or comparable losses of either protein or DNA. Simultaneous treatment with penicillin G and either chloramphenicol or rifampin resulted in reduced levels of killing and the complete inhibition of RNA loss. These findings define a new mechanism of penicillin G-induced killing in the absence of cell wall disruption and suggest a basis for drug-induced antagonism of penicillin G-mediated nonlytic death.

A unified model for the action of inhibitors of cell wall synthesis (23, 26) predicts that in all susceptible bacteria, cell wall antibiotics act by inhibiting the assembly of insoluble peptidoglycan (PG), leading to bacteriostasis. The secondary events are species dependent and can be related to growth rate (2) and medium composition (10). The widely accepted mechanism of penicillin-induced killing is that inhibition of synthesis results in a deregulation of the endogenous PG hydrolases (autolysins), which leads to the destruction of the structural integrity of the PG and ultimately death (23, 26). It is clear, however, that a diverse group of bacteria which are sensitive to penicillin do not lyse, even after prolonged exposure to high concentrations of the drug (3, 19, 23). These nonlytic phenotypes can be grouped into two categories: tolerant strains, which are growth inhibited but lose viability slowly (3, 6, 19, 23), and nonlytic-death strains, which die rapidly after treatment with relatively low doses of, for example, benzylpenicillin (penicillin G). Both classes of nonlytic phenotypes fail to express autolytic activities.

A previously proposed mechanism for nonlytic death predicted that partial hydrolysis of the PG fabric (nicking) would be sufficient to alter the essential structural features of the cell wall and would lead to death (3). However, we were unable to find evidence to support this hypothesis (15).

In this report, we describe results of studies of penicillin-induced nonlytic death in group A streptococcus. Our findings clearly demonstrate that penicillin G induces a rapid, dose-dependent, specific loss of total cellular RNA in the absence of hydrolysis of the cell wall. In addition, we present evidence which suggests that the antagonistic effects of inhibitors of transcription or ribosomal function are associated with impairment of penicillin G-induced RNA hydrolysis. These observations define a mechanism of penicillin G action which can account for the bactericidal nature of the drug in the absence of cellular dissolution.

MATERIALS AND METHODS

Organisms and growth conditions. Group A streptococcus strain 1224 was a clinical isolate obtained from St. Christopher's Hospital for Children, Philadelphia, Pa. *Enterococcus hirae* ATCC 9790 (*Streptococcus faecium*) was obtained from our laboratory stock cultures. All cultures were stored in the lyophilized state. For routine use, inocula were freshly

prepared from frozen glycerol stocks and grown aerobically (24) in chemically defined medium (27) as previously described (15). Growth was monitored turbidimetrically at 675 nm in a Bausch & Lomb, Inc., Spectronic 21 spectrophotometer. The actual optical density values obtained were corrected for deviations in Beer's law as previously described (25) and are expressed here as adjusted optical density units (AOD) (1 AOD = 0.45 μ g [dry weight] of cells per ml and is equivalent to approximately 3.5×10^5 CFU/ml).

Quantitation of growth inhibition and survival. Conventional methods for determining MICs were considered unsatisfactory for these studies because they employ small inocula which are physiologically poorly defined and must have long incubation intervals. Therefore, 50% growth inhibitory concentrations (GIC₅₀s) of each drug for each strain were determined as described previously (17). For these studies we used GIC₅₀s obtained following exposure of exponentially growing cultures (initial AODs, 100 for group A streptococci and 200 for *E. hirae*) to a drug at a time when exponentially growing controls had increased fourfold in turbidity. The potassium penicillin G (1,595 U/mg) used in these experiments was a gift from Wyeth Laboratories, Philadelphia, Pa. Chloramphenicol and rifampin were purchased from Sigma Chemical Co., St. Louis, Mo.

Susceptibility to the lethal effects of antibiotics was determined by using conventional agar overlay techniques (9). Exponentially growing cultures in balanced growth (22) were diluted to the initial turbidities indicated above and exposed to antibiotics at concentrations also indicated. At appropriate times, duplicate 1-ml samples were collected. One was placed into 1 ml of prewarmed (37°C) chemically defined medium, and the other was placed into 1 ml of iced 15% Formalin. Samples collected into medium were immediately processed for determination of CFU, as previously described (15). It should be noted that in this protocol, penicillinase (95 mU/ml) was added to the soft agar to inactivate carry-over penicillin. To control for drug-induced changes in cell chain length, which in some streptococci can lead to spurious results, samples placed in Formalin were used to correct CFU values for variations in cell chain length (18).

Microscopic enumeration of bacteria. The lytic effect of penicillin G treatment on group A streptococci was determined by direct microscopic enumeration of bacteria. In these studies, 1 ml of culture was collected into 1 ml of iced 15% Formalin. Fixed-cell suspensions were counted with a Petroff-Hausser counting chamber. Bacteria present in 20

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squares falling on opposing diagonals of the counting grid were recorded, and the average number of cells present was calculated. All samples were counted in triplicate. In some experiments, samples were coded and counted by two investigators in order to further ensure unbiased results.

Determination of net accumulation of macromolecules. The effects of antibiotic treatment on the net accumulation of PG, RNA, DNA, and protein were determined by radiolabeling techniques described previously (19, 22). These methods, which utilize cultures grown for at least six mass doublings of exponential growth in the presence of appropriate ^3H - or ^{14}C -labeled precursors (^3H]lysine [PG], ^{14}C]uracil [RNA], ^3H]thymidine [DNA], and ^3H]leucine [protein]), were designed to ensure that the radioactivity measured in trichloroacetic acid (TCA) precipitates and insoluble PG residues would reflect the relative amounts of these macromolecules in the cultures (20, 22).

Determinations of hydrolysis and loss of macromolecules. The effects of antibiotic treatment on hydrolysis and loss from cells of RNA, protein, and PG were determined by monitoring the loss of radiolabeled material previously incorporated into the macromolecules. For these chase studies, macromolecules were extensively labeled by growing cultures in the presence of ^{14}C - and ^3H -labeled precursors (described above) for at least six mass doublings of exponential growth. Labeled cells were harvested via filtration (0.45- μm -pore-size membrane filters), washed with two 5-ml volumes of prewarmed (37°C) unlabeled (chase) medium, and suspended from the filters in chase medium by vigorous blending with a Vortex mixer. These cultures were diluted in chase medium and grown for one mass doubling prior to antibiotic treatment. Cultures were exposed to antibiotics by transferring appropriate volumes to prewarmed vessels containing antibiotics. At intervals, duplicate 0.5-ml samples were collected and processed.

(i) **Loss of label from cells.** In experiments which describe the loss of label from cells, cells were either collected on membrane filters (0.45- μm pore size) and immediately washed with two 1-ml volumes of iced medium or collected directly into 5 ml of iced 10% TCA. TCA precipitates were processed as described previously (22). For determination of the amount of label remaining in RNA, the washed membrane filters were dried and counted. To determine the amount of label present in protein, membrane filters were sequentially washed with iced TCA (10%; two 1-ml volumes), hot TCA (10%; 95°C; six 1-ml volumes), iced H_2O (two 1-ml volumes) and iced ethanol (two 1-ml volumes) prior to being dried and counted. The amount of lysine label present in PG was determined by applying a previously described (20) modification of the method of Boothby et al. (1) to cells collected on 0.45- μm -pore-size membrane filters. Samples on membrane filters were washed as described above for determinations of label present in protein. The final ethanol washes were omitted. Washed filters were transferred to plastic cups and digested at 37°C for 90 min with 3 ml of a 1-mg/ml solution of trypsin in 0.1 M sodium phosphate (pH 8). After digestion, filters were placed on a second membrane filter and processed as previously described (1). This protocol effectively reduced the presence of ^{14}C]leucine (protein) in the samples to 7% or less of the amount present in hot-TCA-treated samples. Furthermore, the amount of label present in either protein or PG in untreated control cultures collected and processed on membrane filters was comparable to that found in parallel samples processed by traditional TCA precipitation (22) or modified Boothby (1, 20) procedures.

FIG. 1. Comparison of inhibition of growth (turbidity) and net accumulation of PG, RNA, DNA, and protein after exposure of exponentially growing cultures of group A streptococci to a GIC_{50} (0.1 $\mu\text{g}/\text{ml}$) and a $10 \times \text{GIC}_{50}$ dose of penicillin G. ---, Untreated control cultures. At a turbidity equivalent to 45 μg (dry weight) of cells per ml (time zero), exponentially growing cultures grown in the presence of labeled precursors (see Materials and Methods) were distributed into tubes containing penicillin G. At the times indicated, samples were collected into TCA and processed as described in Materials and Methods. At time zero, 4.0×10^4 , 3.2×10^3 , 1.2×10^4 , and 2.1×10^4 dpm per sample were present in DNA, RNA, protein, and PG, respectively.

(ii) **Hydrolysis studies.** To determine the kinetics of hydrolysis, samples were collected into 5 ml of 95°C H_2O and extracted at 95°C for 15 min. Unlabeled carrier cells were added (0.5 ml of a suspension at an AOD of 1,000), and the samples were chilled for at least 30 min in an ice bath. Chilled samples were collected on membrane filters (0.45- μm pore size) and washed sequentially with two 1-ml volumes of iced H_2O and two 1-ml volumes of iced ethanol. Filters were dried and counted.

Determinations of radioactivity. All samples dried on filters were transferred to filmware scintillation vials. Scintillation cocktail (Scinti Verse E; Fisher Scientific Co., Pittsburgh, Pa.) was added and counted in a scintillation spectrophotometer. Corrections for quench and overlap of ^{14}C counts into the ^3H channel were made by an appropriately programmed computer. All results were expressed as disintegrations per minute.

RESULTS

Effects of penicillin G on net accumulation of macromolecules. In a previous report (19) we described the sequential

FIG. 2. Comparison of the kinetics of hydrolysis of RNA (A) and protein (D) and the loss of hydrolysis products from cells and TCA-precipitable material (RNA [B and C] and protein [E and F]) after exposure of exponentially growing cultures of group A streptococci to increasing concentrations of penicillin G, as measured by label-chase techniques. Parallel cultures were extensively labeled with [14 C]uracil (RNA) or [14 C]leucine (protein) prior to harvesting and transfer to chase medium. In this experiment, quantitation of [14 C]leucine remaining with cells (E) was done by the method described for RNA; there was a medium wash only. All other samples were collected and processed as indicated in Materials and Methods. At time zero, samples extracted with H_2O had 3.1×10^3 and 3.7×10^3 dpm per sample, samples washed with medium had 4.1×10^3 and 4.8×10^3 dpm per sample, and TCA-precipitated samples had 7.5×10^3 and 5.5×10^3 dpm per sample in RNA and protein, respectively.

and dose-dependent inhibition of PG, RNA, protein, and DNA following exposure of exponentially growing cultures of tolerant streptococci to penicillin G. A similar analysis of group A streptococci (a nonlytic-death phenotype) revealed a quite different pattern (Fig. 1). Exposure of exponentially growing cultures to a GIC_{50} or a saturating dose ($10 \times GIC_{50}$) of penicillin G resulted in reductions in culture turbidity suggestive of bacterial lysis. However, these losses were accompanied by a loss of label only from TCA-precipitable RNA and not from protein, DNA, or PG. In addition, microscopic enumeration of bacteria present in penicillin G-treated cultures revealed no correlation between the observed losses of culture turbidity and reductions in cell number. For example, cultures which had undergone an 81% reduction in turbidity as a result of prolonged exposure (6 h) to a high ($100 \times GIC_{50}$) concentration of penicillin G contained 92% of the cells present when the culture was at its maximum turbidity (45 min after exposure).

Penicillin G inhibited the accumulation of PG more effectively than did the other macromolecules studied (Fig. 1). Exposure of cultures to either concentration of penicillin G inhibited the accumulation of PG completely and affected the accumulation of DNA to a similar extent. Interestingly, growth, accumulation of RNA and protein (Fig. 1), and losses of viability were affected in a dose-dependent manner; i.e., at a time equivalent to two mass doublings of the control cultures, approximately 70% of cultures exposed to a GIC_{50} dose remained viable, while less than 10% of cultures treated with the high dose were able to form colonies.

Effects of penicillin G on hydrolysis and loss of macromolecules. The effects of penicillin G treatment on the integrity of macromolecules were examined by quantitating previ-

ously incorporated radiolabel which was either retained on membrane filters or remained TCA precipitable. The pattern of [14 C]uracil loss from cells extracted with $95^\circ C$ H_2O reveals the rapid and dose-dependent conversion of label to an extractable form, i.e., RNA hydrolysis (Fig. 2A). Release of label incorporated in RNA from cells collected directly on filters (Fig. 2B) was exponential. At all concentrations of penicillin G, hydrolysis of RNA (Fig. 2A) preceded the loss of label from cells (Fig. 2B). Label in RNA which could be precipitated by TCA was also reduced at an exponential rate and in a dose-dependent manner (Fig. 2C).

The patterns of hydrolysis and loss of protein are also shown (Fig. 2D through F). Results of these studies show the limited effect of penicillin G on this class of molecules. For example, at the highest drug concentration and after an extended incubation interval (6 h), a maximum of approximately 40% of the label previously incorporated in protein was released (Fig. 2E) compared with a greater than 90% loss of label from RNA (Fig. 2B). In addition, approximately 80% of the [14 C]leucine in protein remained TCA precipitable (Fig. 2F).

The effects of exposure to comparable concentrations ($10 \times GIC_{50}$) of penicillin G on loss of turbidity, viability, PG, RNA, and protein in group A streptococci along with those observed for *E. hirae* (a lytic phenotype) are presented in Fig. 3. Exposure to this concentration of penicillin G resulted in a loss of turbidity and a rapid loss of viability in cultures of both bacteria (Fig. 3A). However, only in cultures of *E. hirae* were these reductions accompanied by comparable losses of label from all three macromolecules (Fig. 3B through D), i.e., cell lysis. Microscopic enumeration of bacteria (data not shown) confirmed the lytic process.

FIG. 3. Comparison of growth inhibition (AOD); loss of PG ($[^3\text{H}]$ lysine), RNA ($[^3\text{H}]$ uracil), and protein ($[^3\text{H}]$ leucine); and viability (survival) after exposure of exponentially growing cultures of *E. hirae* and group A streptococci (GAS) to a $10 \times \text{GIC}_{50}$ (25 and $1 \mu\text{g/ml}$, respectively) dose of penicillin G. ---, Untreated control cultures, in which *E. hirae* doubled in mass every 42 min and group A streptococci doubled in mass every 36 min (A). Symbols (B through D): ○ and △, untreated control cultures; ● and ▲, treated cultures. For culturing, labeling, and processing conditions, see the legend for Fig. 2 and Materials and Methods. In these experiments, cells were suspended in chase medium at a turbidity equivalent to 45 and $22.5 \mu\text{g}$ (dry weight) of cells per ml for *E. hirae* and group A streptococci, respectively, and grown as described in the legend to Fig. 2. Symbols (A): ◇ and ◆, viability (percent CFU of the time zero control) of *E. hirae* and group A streptococci, respectively. At time zero, *E. hirae* contained 1.3×10^4 , 5.9×10^3 , and 1×10^4 dpm per sample and group A streptococcus cultures contained 1.2×10^4 , 8.9×10^3 , and 4×10^4 dpm per sample in PG, RNA, and protein, respectively.

Studies with antibiotic combinations. Active bacterial growth is considered a prerequisite for the expression of penicillin action. Consequently, the simultaneous treatment of bacteria with bacteriostatic drugs significantly inhibits the killing action of penicillin G (7). The mechanism of these antagonistic effects has been attributed to the inhibition of expression of autolytic activity. In a previous report (17), we described the effects of the simultaneous treatment of group A streptococci with chloramphenicol and penicillin G, and we now extend these observations to include rifampin (Fig. 4). A portion of the results obtained with chloramphenicol

FIG. 4. Effects of simultaneous exposure to rifampin ($10 \times \text{GIC}_{50}$, $0.25 \mu\text{g/ml}$) and penicillin G ($10 \times \text{GIC}_{50}$, $1 \mu\text{g/ml}$) on growth (AOD) and viability (survival) and comparison of the effects of simultaneous exposure to either rifampin-penicillin G or chloramphenicol ($10 \times \text{GIC}_{50}$, $20 \mu\text{g/ml}$) and penicillin G ($10 \times \text{GIC}_{50}$) on loss of RNA ($[^3\text{H}]$ uracil) and PG ($[^3\text{H}]$ lysine) in exponentially growing cultures of group A streptococci. ---, Untreated control cultures. For experimental details, see the legend to Fig. 2 and Material and Methods. Symbols (A): ◇ and ◆, viability (percent CFU of time zero control) of penicillin G and rifampin-penicillin G-treated cultures, respectively. At time zero, control cultures from experiments with rifampin had 3.5×10^3 and 7.5×10^3 dpm per sample, while those from chloramphenicol studies contained 2.7×10^3 and 1.5×10^4 dpm per sample in RNA and PG, respectively.

are presented for comparative purposes. Combinations of rifampin and penicillin G had a marked additive effect on growth inhibition, while the addition of rifampin (or chloramphenicol; data not shown) significantly antagonized penicillin G-induced losses in viability (Fig. 4A). Combinations of either rifampin or chloramphenicol and penicillin G dramatically inhibited the penicillin G-induced loss of RNA (Fig. 4B). Interestingly, rifampin and chloramphenicol, both alone and in combination with penicillin G, produced quite different effects on PG. Cultures exposed only to rifampin showed an increased level of label in PG (Fig. 4C). Combinations of rifampin and penicillin G resulted in neither an increase nor a decrease in cell wall-associated label. In contrast, exposure to chloramphenicol did not produce a significant increase in $[^3\text{H}]$ lysine in the cell wall. However, combinations of chloramphenicol and penicillin G induced a rather rapid and extensive release of label previously incorporated in PG (Fig. 4C).

DISCUSSION

The bactericidal mechanism of action of penicillin in this nonlytic-death phenotype is not associated with cellular lysis or extensive loss of the cell wall (insoluble PG). Results of experiments described here (Figs. 1, 3, and 4) and elsewhere (15) provide no evidence to support an association between observed losses of culture turbidity and hydrolysis of PG. Studies involving direct microscopic enumeration of bacteria confirm these findings. In addition, exposures to relatively low and high doses of penicillin G showed a concentration-dependent effect on killing, on accumulation and hydrolysis of RNA, and, to a lesser extent, on protein (Fig. 1 and 2) but no effect on accumulation of PG or DNA (Fig. 1). Comparison of the kinetics of RNA hydrolysis with those of the loss of label from cells (Fig. 2A and B) indicates the presence of an at least partially intact permeability barrier. The kinetics of protein hydrolysis (Fig. 2D) suggests that a subset of proteins is rapidly converted to an extractable form with little if any subsequent dissolution (Fig. 2F). In a previous report (17), we presented additional data describing the effects of penicillin G on PG and DNA. Also, as with tolerant streptococci (20), the cell walls of group A streptococci are apparently not subject to turnover (Fig. 3B [17]).

The experiments described here were conducted with only one group A streptococcal strain. However, we have surveyed eight additional clinical isolates and one laboratory strain (R. C. Lancefield strain S43/192/1, obtained from R. E. Kessler, Bristol-Myers Co.). In all cases, we observed results consistent with those described above. Two previous reports (12, 21) have described the loss of RNA after exposure of bacteria to beta-lactam antibiotics. However, both studies were conducted with gram-negative enteric bacilli, which, under certain conditions, will lyse when exposed to the appropriate beta-lactam.

In group A streptococcus strain 1224, the antagonistic effects of inhibitors of either transcription (rifampin) or ribosomal function (chloramphenicol) on the bactericidal action of penicillin G are not mediated by preventing the expression of autolytic activity (Fig. 4) (17). This strain does elaborate a PG hydrolase, as is evidenced in this report by the chloramphenicol-penicillin G-induced solubilization of PG (Fig. 4C) and described previously (15). However, expression of this activity is apparently quite different from that found in other bacteria (10, 28) (Fig. 3), since losses in excess of 50% of the PG were not sufficient to disrupt the structural integrity of the cell wall; e.g., this drug combination did not lead to a loss of RNA (Fig. 4C) or reductions in turbidity (17).

The similarity of the chloramphenicol-penicillin G induction of PG hydrolysis described here for group A streptococci and that reported for *Escherichia coli* (disruption of the stringent response [10]) has been discussed previously (17). The inability of rifampin-penicillin G combinations to produce a similar effect is likely related to the site of action of rifampin; i.e., rifampin impairs RNA polymerase activity, does not directly affect ribosomal function, and consequently does not disrupt the stringent regulation of PG synthesis (10).

An additional difference between rifampin and chloramphenicol treatment of group A streptococci was observed. Rifampin treatment resulted in an increase in label in PG, which is reminiscent of drug-induced cell wall thickening in other streptococci reported previously (4, 5, 13, 14). Since in these experiments label was not present in the medium and cellular pools had been depleted prior to exposure to the

antibiotics, [³H]lysine previously incorporated in protein apparently was transferred to PG. Why rifampin, and not chloramphenicol, stimulated an increase in the amount of label present in PG is not known.

In summary, the mechanism of action of beta-lactam antibiotics needs to be broadened to include induction of RNA hydrolysis. Also, bacteria classified as lytic phenotypes solely on the basis of antibiotic-induced loss of culture turbidity may need to be reevaluated with regard to RNA hydrolysis versus solubilization of the cell wall. On the basis of results presented here (Fig. 4) and previously (17), rifampin- and chloramphenicol-induced reductions of penicillin G-associated killing correlate best with the inhibition of RNA hydrolysis. Studies of the effects of cell wall antibiotics on RNA in *Streptococcus mutans* (16, 19; unpublished results) demonstrate that in this tolerant streptococci, exposure to penicillin G, for example, does not induce any significant hydrolysis of RNA. Furthermore, experiments conducted with both derived and naturally occurring tolerant bacteria (6, 8, 11) have shown that antibiotic treatment of these strains does not lead to loss of culture turbidity; i.e., exposure of tolerant bacteria to cell wall antibiotics does not lead to a significant hydrolysis of either PG or RNA.

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