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Lethal Effect of a Heterologous Murein Hydrolase on Penicillin-Treated *Streptococcus sanguis*

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Nine strains of *Streptococcus sanguis* exhibited tolerance to benzylpenicillin: the growth of each strain was susceptible to penicillin with minimal inhibitory concentrations of 0.1 μg/ml or lower, but the bacteriolytic and bactericidal effects were limited in each case. The tolerance of these bacteria was also reflected in the large discrepancies between the minimal inhibitory and minimal bactericidal concentrations for benzylpenicillin. The hypothesis that a natural deficiency of endogenous murein hydrolase (autolysin) in this species accounts for the penicillin tolerance was tested by using a heterologous murein hydrolase, the C-phage-associated lysin. In seven of the strains, addition of the lysin to the culture together with penicillin or other cell wall inhibitors resulted in lysis and rapid loss of viability. The enzyme alone did not appreciably affect normally growing cultures. The irreversible effects of penicillin plus lysin were drastically reduced in the presence of the bacteriostatic agents chloramphenicol and cerulenin. Speculations based on experiments are presented for the mechanisms by which penicillin treatment sensitizes these bacteria to an exogenous lytic enzyme. Similar phenomena requiring cooperation of host factors and penicillin may occur during infection, since somewhat similar although less pronounced results were obtained by addition of human lysozyme to penicillin-treated *S. sanguis*.

The importance of bacterial murein hydrolases (autolysins) in mediating the bacteriolytic effects of penicillin has been documented in several species of gram-positive bacteria (26, 29, 30). Autolysin-defective mutants or physiological variants are as susceptible to the growth-inhibitory effects of penicillin as the wild-type strains, but are resistant to the lytic and to some extent the cytocidal effects of the drug (13, 21, 29). The term "antibiotic tolerance" was originally coined in reference to this phenomenon, which was first described in autolysin-defective laboratory strains of pneumococci (29, 30). Recently this term has been used to describe clinical isolates from several species of bacteria exhibiting relative resistance to the irreversible effects of penicillin and other beta-lactam antibiotics (i.e., minimal bactericidal concentrations [MBCs] many times higher than minimal inhibitory concentrations [MICs]; 27).

In an earlier communication we described a naturally occurring penicillin-tolerant bacterium, *Streptococcus sanguis* strain Wicky (17). The response to penicillin of eight other strains, including five isolated from endocarditis patients, has since been investigated. In all cases, the organisms were susceptible to relatively low concentrations of penicillin, the MICs ranging from 0.006 to 0.1 μg/ml, but little or no bacteriolysis was detected with any of the strains. Similarly, reductions in viable titer occurred at extremely slow rates. The strains also showed large discrepancies between benzylpenicillin MICs and MBCs.

Since the response of this species to penicillin is very similar to that of bacteria with suppressed autolytic systems (26, 29, 30), it appeared possible that the apparent natural deficiency in autolysin of this species (17) may account for the absence of the irreversible effects of penicillin. In this communication, we report that addition of penicillin (or other cell wall inhibitors) to the majority of these strains sensitized the bacteria to the action of a heterologous murein hydrolase, causing rapid lysis and cell death.

MATERIALS AND METHODS

Cultures. The strains of *S. sanguis* used in these studies included the laboratory strains Wicky, Challis, Blackburn (from Dennis Perry, Northwestern University Medical-Dental Schools, Chicago, Ill.), and F90A (from Maclyn McCarty of this university). Five clinical isolates from New York Hospital (Table 1), identified as *S. sanguis* by biochemical and physiological tests, were kindly donated by Richard B. Roberts (Cornell University Medical Center). Cultures were grown in Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.) or were grown overnight in brain heart infusion (Difco) and diluted 2% (vol/vol) into C+Y medium (containing acid-hydrolyzed casein; 20)

TABLE 1. *Susceptibility of nine strains of S. sanguis to benzylpenicillin*

Strain	MIC[a] (μg/ml)	MBC[a] (μg/ml)		Viable titer[b] (% survival at 20 × MIC)		Lysis by penicillin + C-phage-associated lysin[c]
		24 h	48 h	3 h	20 h	
Wicky	0.05	0.01	100	75	12	+
Challis	0.02	0.08	10	70	10	+
Blackburn	0.02	0.08	100	91	3	+
F90A	0.02	0.08	10	87	15	±
Ws	0.05	0.1	100	82	ND[d]	+
Yg 4295	0.02	0.04	100	82	ND	+
Re 3852	0.02	0.04	100	65	7	+
Hn 995[e]	0.006	0.012	0.03	25	3	−
On 1899[e]	0.15	0.3	100	77	15	−

[a] The MIC was recorded as the lowest concentration of benzylpenicillin preventing visible growth after 24 h at 37°C in a tube dilution assay. MBC was defined as the lowest concentration causing 99.9% killing in 24 h and was measured as described in the text. The number of survivors was recorded after 24 and 48 h of incubation, respectively, of the MBC plates.

[b] Benzylpenicillin (20 × MIC) was added to exponentially growing cultures of *S. sanguis* at cell densities of 4×10^7 per ml. The percentage of surviving cells after 3 and 20 h of antibiotic treatment was determined as described in the text.

[c] Bacteria at cell densities of 4×10^7 CFU per ml were exposed to 200 × MIC of penicillin plus 1.5 U of the lysin per ml. Lysis was determined by nephelometry. Lysis was considered positive if the turbidity reading after 2 h was less than half of the initial reading.

[d] Not determined.

[e] Antigenic extracts of these two strains failed to react with anti-F90A serum.

buffered at pH 7.6 with 0.05 M sodium phosphate buffer and supplemented with 0.1% of the dialyzable components of yeast extract (Difco). The MICs of benzylpenicillin did not vary significantly between the two media. Bacteria were grown without aeration at 37°C, usually in 10 ml of medium in 18-mm-wide tubes. Growth of the cultures was monitored by measuring the light scattering (Coleman nephelometer; 28) or by determining the turbidity at 550 nm (1-cm cuvettes; Zeiss spectrophotometer). Viable counts (colony-forming units [CFU] per milliliter) were determined by routine plating procedures on commercially available 5% sheep blood agar plates. In a few experiments, the bacteria were "dechained" by vigorous stirring (2 × 2 min) with a tissue homogenizer (Tekmar Co., Cincinnati, Ohio) before plating.

Antiserum prepared against strain F90A (group H reference strain) was kindly provided by Maclyn McCarty. Antigens from the nine strains (grown in THB) were extracted by the autoclave method of Rantz and Randall (25) and tested against the antiserum by capillary precipitation assay and single-dimension agarose gel diffusion.

Antibiotics. The antibiotics used in these experiments were: benzylpenicillin (Eli Lilly & Co., Indianapolis, Ind.); 3-fluoro-2-deutero-D-alanine and a derivative of D-cycloserine {(D-4-[(2-oxo-3-pentene-4-yl)-amino]-3-isoxazolidinone, sodium salt}, used in combination. Both of these drugs and cefoxitin were obtained from Merck Research Laboratories (Rahway, N.J.). Other drugs used were cerulenin (Makor Chemicals, Jerusalem, Israel); chloramphenicol (Sigma Chemical Co., St. Louis, Mo.); piperacillin (Lederle Laboratories, Pearl River, N.Y.); oxacillin, cephaloridine, cephalexin, and cephalothin (Bristol Laboratories, Syracuse, N.Y.); dicloxacillin (Wyeth Laboratories, Philadelphia, Pa.); and mecillinam (Leo Co., Ballerup, Denmark). Stock solutions (usually in distilled water) were stored at −20°C. Penicillinase was purchased from Calbiochem (La Jolla, Calif.).

Lytic enzymes. Human urinary lysozyme was purchased from Worthington Biochemicals Corp. (Freehold, N.J.). Crude C-phage-associated lysin was prepared from 4-liter cultures of C1 phage-infected group C streptococcal strain 26RP66; the enzyme was inactivated with sodium tetrathionate and reactivated with dithiothreitol by the method of Fischetti et al. (9).

Susceptibility of the strains to benzylpenicillin. The susceptibility of each of the nine strains to benzylpenicillin was measured in a tube dilution assay. Two-fold serial dilutions of benzylpenicillin were made in tubes containing 0.5 ml of THB. An equal volume of a cell suspension (0.5×10^6 to 1×10^6 CFU per ml, prepared from an 18-h culture) was then added, and the tubes were incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration of antibiotic preventing visible growth. The MBC (99.9% killing in 24 h) was determined by streaking 10-μl samples from the MIC tubes on plates containing 10^2 U of penicillinase per ml. The addition of penicillinase was considered important to prevent inhibition of growth of the survivors by residual penicillin.

Biosynthetic labeling of cell walls. The bacterial cell walls of the Wicky strain were radioactively labeled (in the murein and polysaccharide moieties) with *N*-acetyl-D-[1-^{3}H]glucosamine ([^{3}H]GlcNAc; 4.7 Ci/mol; Amersham Corp., Arlington Heights, Ill.), 5 μCi and 0.3 μg per ml of growth medium (17).

The [^{3}H]GlcNAc was added to an exponentially growing culture at a cell density of 10^7 CFU per ml, and growth continued for 60 min. The bacteria were harvested by centrifugation, washed once, and transferred to isotope-free medium for 15 min of incubation

at 37°C. An antibiotic was added to a 10-ml portion of the culture (4×10^7 CFU/ml), and incubation continued for 30 min unless otherwise stated. Cell walls were prepared from the cell pellets (combined with cells from 200 ml of unlabeled cultures which had been identically treated) by a published procedure (22). The cell walls were often extracted with 2% sodium dodecyl sulfate at 80°C for 30 min, followed by extensive washing.

Determination of lysin activity. Enzyme activity was measured in the following manner: [^{3}H]GlcNAc-labeled cell walls (2.5 μg and 2.5×10^4 dpm) were mixed with a portion of the enzyme in a final volume of 250 μl of lysis buffer (0.05 M sodium phosphate buffer [pH 6.1] containing 5 mM mercaptoethanol and 5 mM ethylenediaminetetraacetate [EDTA]). After incubation at 37°C, 50 μl of 10% formaldehyde and 25 μl of 4% bovine serum albumin (Armour fraction IV) solution were added, and the unhydrolyzed cell walls were removed by centrifugation at 12,000 × g for 10 min in an Eppendorf microcentrifuge (Brinkmann Co., Teaneck, N.J.). Radioactivity released into the supernatant fluid was assayed by placing a 200-μl sample into 5 ml of Biofluor scintillation fluid (New England Nuclear Corp., Boston, Mass.) and counting in a Mark II scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). One unit of lysin was arbitrarily defined as the amount needed to release 50% of the label from the cell walls after 15 min at 37°C.

Enzymatic lysis of whole cells. Suspensions of cells (4×10^7 CFU/ml) in lysis buffer or growth medium were incubated at 37°C with 0.5 to 1.5 U of lysin per ml, control cultures receiving an equal volume of 50 mM dithiothreitol instead of lysin. Growth and lysis of the cultures were monitored by measuring the light scattering or the turbidity as described above. The radioactivity released from bacteria prelabeled with [^{3}H]GlcNAc was determined with a 200-μl portion of the supernatant fluids (see above).

Extraction procedures. The lipids were extracted from cell pellets of strain Wicky by the procedure of Ames (1). Lipoteichoic acid (LTA) was phenol extracted from a cell pellet (from a 16-h culture grown in 3 liters of THB), precipitated, and washed free of phenol with ethanol and diethyl ether as described by Elliot et al. (8). The precipitated material was dissolved in 0.5 ml of 0.05 M tris(hydroxymethyl)aminomethane maleate buffer (pH 7) (TM) and treated with 40 μg of ribonuclease A, 190 U of T_1 ribonuclease (Sigma Chemical Co), and 10 μg of deoxyribonuclease I for 3 h at 37°C. The nuclease-treated material was then applied to a column of Bio-Gel A-5 M (Bio-Rad Laboratories, Richmond, Calif.; 1.5 by 50 cm) and eluted with TM buffer. Fractions of 1 ml were collected, and aliquots were assayed for LTA content by the serological method of Ofek et al. (23). LTA-containing fractions were pooled, dialyzed against distilled water, and lyophilized.

Toluene and diethyl ether-treatment of whole cells. Samples from a culture of Wicky (4×10^7 CFU/ml) were mixed on a Vortex mixer for 10 s with 5% toluene (vol/vol) or an equal volume of ether and placed on ice for 10 min. The organic phase was removed; then the cells were harvested by centrifugation (17,000 × g) and washed twice with medium. Pellets were resuspended in the original volume of medium before testing for susceptibility to lysin. The treated cells appeared intact under phase contrast (Zeiss microscope fitted with a Planachromat 100/1.25 phase-contrast oil immersion objective).

RESULTS

Susceptibility of *S. sanguis* to benzylpenicillin. Figure 1 shows the effect of benzylpenicillin on the growth and viability of three representative strains of *S. sanguis*. Six additional strains used in this investigation exhibited virtually identical responses to the antibiotic (17), i.e., inhibition of growth without significant lysis and with an extremely slow decline in viable titer indicative of the tolerance of this species to penicillin.

In apparent contradiction to the slow loss of viability demonstrated in the killing curves, determination of the MBCs of benzylpenicillin usually gave values near or identical to the MICs if the standard clinical assay was used: incubation of cultures with low density of cells (5×10^5 CFU/ml) for 24 h with the antibiotic followed by streaking aliquots on the surface of blood agar plates. The MBC plates are then incubated

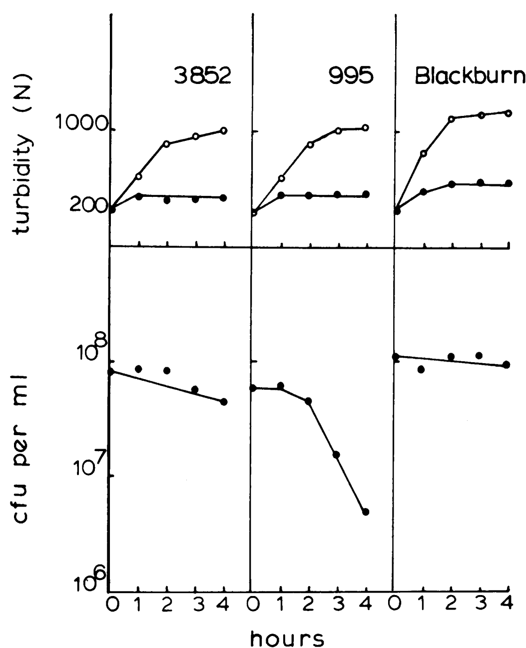

FIG. 1. *Effect of benzylpenicillin on the growth and viability of three strains of S. sanguis in THB medium. Growth was monitored by nephelometry. Viable counts were determined on blood agar plates as described in the text after a minimum incubation time of 48 h. Symbols:* (○) *control;* (●) *plus benzylpenicillin (20 × MIC).*

at 37°C for 24 h. However, we found that upon prolonged incubation of the same plates (up to 72 h), a fairly large portion of the treated bacteria survived to form colonies (Table 1), resulting in a dissociation of MIC and MBC values in all but one (Hn 995) of the strains. The MBC value was also affected by the growth medium used in the susceptibility assay, tending to be much higher with complex medium (THB) than with C+Y medium. However, this may merely reflect the lower percentage of survival of these bacteria in late stationary phase in the latter medium (unpublished observation).

Sensitization of *S. sanguis* to C-phage-associated lysin (lysin) in vivo. Growing cultures of *S. sanguis* strain Wicky were resistant to lysin added to the growth medium (up to 5 U/ml) but were lysed by the same enzyme in buffer. In addition, we noted that penicillin pretreatment of the cells stimulated lysin-induced protoplast formation in hypertonic buffer. These observations suggested that penicillin treatment might sensitize *S. sanguis* to exogenous lysin added to the growth medium. This was in fact found to be the case (Fig. 2). Addition of lysin to the medium of penicillin-inhibited strain Wicky induced cellular lysis and death (Fig. 2, curve 6), although such irreversible effects were not observed with either penicillin or lysin alone (Fig. 2, curves 2 and 5). Lysis of the bacteria by penicillin plus enzyme could be demonstrated in THB, C+Y (as shown), or brain heart infusion medium (not shown). Pretreatment of cultures with high concentrations (20 × MIC) of bacteriostatic antibiotics such as cerulenin (specific inhibitor of fatty acid synthesis; 24) or chloramphenicol partially antagonized both the bacteriolytic and bactericidal effects of penicillin plus lysin (Fig. 2, curve 8).

The laboratory strains and the clinically isolated strains were all treated in growth medium (THB) with the combination of penicillin (200 × MIC) and lysin (1.5 U). Of the nine strains, six (Challis, Blackburn, Wicky, Re 3852, Ws, and Yg 4295) were completely lysed within 2 h of incubation at 37°C (but not by lysin alone) (Table 1); one (F90A) was partially lysed in the same time period; and two (On 1899 and Hn 995) were not lysed at all and were also resistant to the enzyme when suspended in lysis buffer (data not shown). The antigenic extracts (see Materials and Methods) prepared from these latter two strains were the only ones which failed to yield a visible precipitate with anti-F90A serum. The differences in the responses of the strains to the antibiotic and enzyme mixture as well as the antigenic differences probably reflect heterogeneity within the species which has been well documented elsewhere (5, 6). Interestingly, susceptibility or resistance in lysis buffer to the

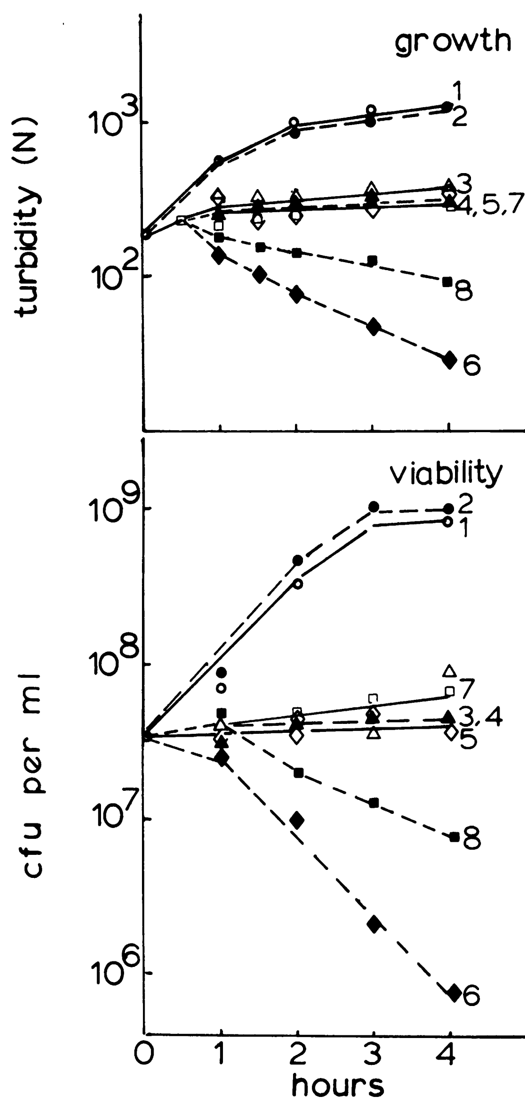

FIG. 2. Bacteriolytic and bactericidal effects of benzylpenicillin treatment of S. sanguis strain Wicky in the presence of C-phage-associated lysin and antagonism of these effects by cerulenin pretreatment of the culture. Ten-milliliter samples of an exponentially growing culture in C+Y medium received benzylpenicillin (1 μg/ml) at zero time, followed by addition of 150 μl of lysin (1.5 U/ml). Control cultures (minus lysin) received 150 μl of 50 mM dithiothreitol. Cerulenin was added 5 min before penicillin. The numbers refer to the experimental samples as follows: (1) control; (2) plus lysin alone; (3) plus cerulenin; (4) plus cerulenin and lysin; (5) plus penicillin; (6) plus penicillin and lysin; (7) plus cerulenin and penicillin; and (8) plus cerulenin, penicillin, and lysin. Growth, lysis, and viability were determined as described in the legend to Fig. 1.

phage-associated lysin was one of the attributes used to classify strains of *S. sanguis* into types I, II, and heterogeneous type by R. Cole and coworkers (5).

Specificity of sensitization to C phage-associated lysin. Figure 3 illustrates the close correlation between the extent of lysis and killing in the Wicky strain by the enzyme and the concentration of penicillin. Significant sensitization to the lysin in vivo (detected either as decrease in light scattering or loss of viability during a 4-h period at 37°C) required relatively high concentrations of the drug. In addition, lysis commenced after a considerable lag (30 min or more, depending on the dose). Therefore, the dose response and kinetics of lysis by the enzyme plus penicillin are distinct from those of the previously reported secretion phenomenon (16, 17); however, sensitization to lysin (like the drug-induced secretion) seemed to be specifically related to inhibition of murein synthesis as indicated below.

Several antibiotics were tested at 5 × MIC or more for their capacity to sensitize the Wicky strain to the lysin (Fig. 4). Several cephalosporins and bacitracin were even more effective sensitizing agents than penicillin when compared on the basis of MIC values. Other cell wall inhibitors that were also active were cephalothin, oxacillin, dicloxacillin, and a combination of fluoro-D-alanine and a D-cycloserine derivative (data not shown). Two notable exceptions were the beta-lactams mecillinam and piperacillin, which were weakly active. These two antibiotics were tested at 20 × MIC for their ability to stimulate secretion of glycerol-labeled macromolecules as described elsewhere (17); both were active in this respect, but less so than penicillin or the cephalosporins used (data not shown).

Specific inhibitors of ribonucleic acid and protein syntheses (rifampin and chloramphenicol) were virtually inactive in sensitizing the bacteria to enzymatic lysis. Chloramphenicol actually antagonized penicillin-mediated sensitization, as did cerulenin (Fig. 2). The mechanism for the low level of sensitization obtained with mitomycin C, a specific inhibitor of deoxyribonucleic acid synthesis, is not clear at the present time.

Mechanism of sensitization to the lysin. The following set of experiments was done in an attempt to explore the mechanism of the sensitization phenomenon (Table 2). Methods other than antibiotic treatment were tested for possible sensitizing activity. Freezing and thawing a culture caused "transient" susceptibility to the lysin (i.e., an initial reduction in turbidity followed by resumption of growth). Sodium deoxycholate and EDTA [but not ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid] were more or less as effective sensitizing agents as penicillin. Brief extraction with ether or toluene was by far the most effective treatment in

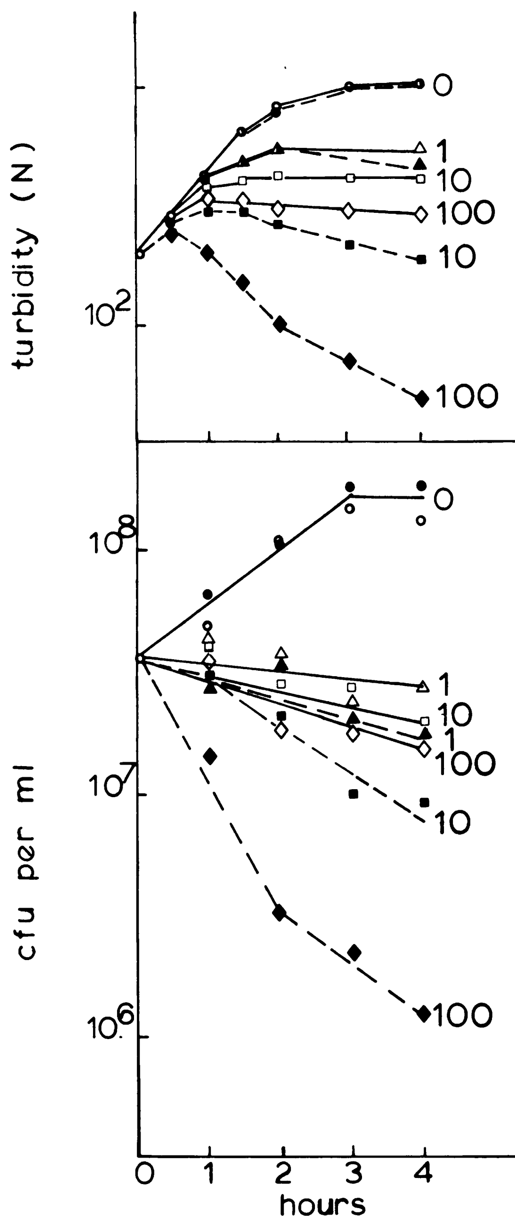

FIG. 3. *C-phage-associated lysin-mediated lysis and killing of S. sanguis strain Wicky with various concentrations of benzylpenicillin ranging from 1 to 100 × MIC. The numbers represent the concentration of antibiotic expressed as multiples of MIC values. Symbols: (——) control samples minus enzyme; (- - -) plus lysin (1.5 U/ml). Growth, lysis, and viable titers were measured as described in the legend to Fig. 1.*

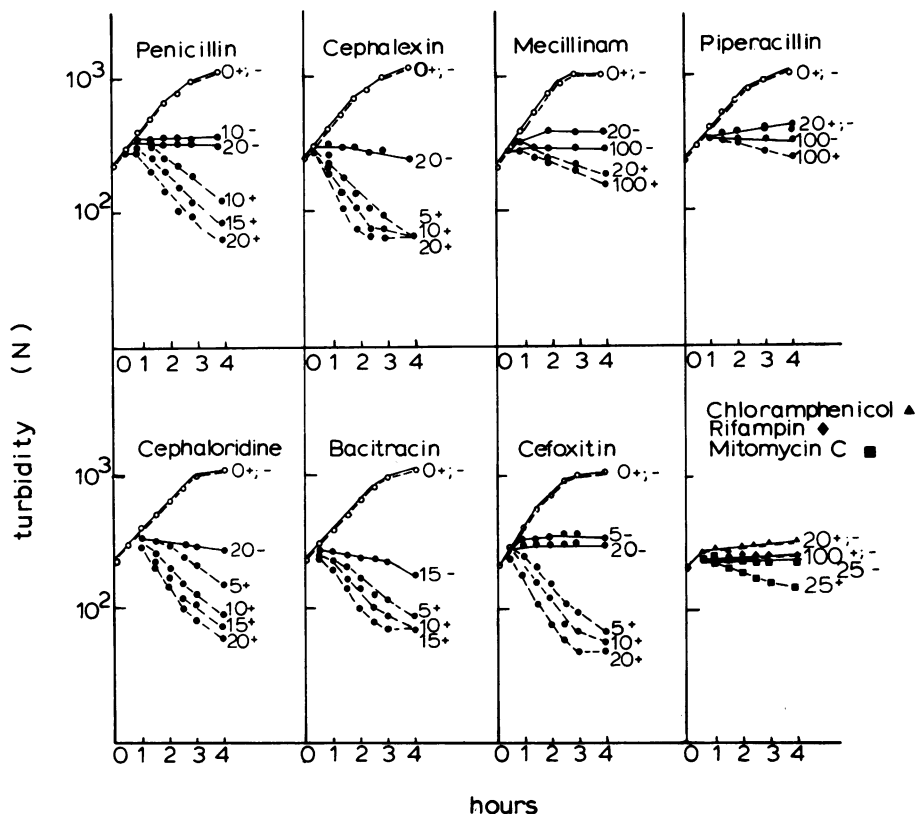

FIG. 4. Response of S. sanguis strain Wicky to various metabolic inhibitors in the presence of C-phage-associated lysin. Numbers represent the concentrations of the drugs expressed in multiples of the MIC values. The MICs for the antibiotics (determined as described in the text) were as follows: benzylpenicillin, 0.05 μg/ml; cephalexin, 10 μg/ml; mecillinam, 8 μg/ml; piperacillin, 0.05 μg/ml; cephaloridine, 0.02 μg/ml; bacitracin, 25 μg/ml; cefoxitin, 3 μg/ml; chloramphenicol, 3 μg/ml; rifampin, 0.1 μg/ml; and mitomycin C, 0.1 μg/ml. Symbols: (—, -) control samples minus enzyme; (- - -, +) plus enzyme (1.5 U/ml). Growth and lysis were determined as described in the legend to Fig. 1.

eliciting sensitization: virtually complete lysis occurred within 30 min of incubation with the lysin.

A series of experiments was done to measure the susceptibility of cells (prelabeled in their cell walls with [^{3}H]GlcNAc and subsequently treated with penicillin or chloramphenicol for 30 min in growth medium) to a limiting amount of lysin in buffer. Differences in the rates of lysis were detected (order of decreasing rates: penicillin-treated > control > chloramphenicol-treated bacteria). However, when the treated and control cells were subsequently exposed to a cycle of freezing and thawing, they all lysed with comparable rates during incubation with lysin (Fig. 5A).

In some experiments, the degree and rate of lysis (measured as drop in turbidity or release of [^{3}H]uracil-labeled cellular material) was compared with the degree and rate of cell wall degradation (measured by determining the release of radioactive cell wall fragments). The findings indicate that virtually complete lysis (as indicated by optical clearing of the suspensions or by the release of over 80% of intracellular, i.e., [^{3}H]uracil-containing, radioactivity) was accompanied by a relatively limited degree of cell wall degradation (30% of cell wall label released).

In another set of experiments, we compared cell walls (purified by hot sodium dodecyl sulfate extraction) prepared from control and penicillin-treated cells for their susceptibility to limiting concentrations of the phage-associated lysin. The rates of hydrolysis (Fig. 5B) observed were virtually identical, indicating that the penicillin treatment did not cause the production of a murein that would be grossly hypersensitive to the action of the lytic enzyme. One should re-

TABLE 2. *Response of S. sanguis strain Wicky to C-phage-associated lysin: effect of various sensitizing agents*

Pretreatment[a]	Degree of lysis (% reduction in initial turbidity)			
	0.5 h		1 h	
	− Lysin	+ Lysin	− Lysin	+ Lysin
None	—[b]	—	—	—
Benzylpenicillin (10 μg/ml)	0	30	0	68
Deoxycholate (0.02%)	0	30	0	39
EDTA (5 mM)	0	15	0	36
EGTA[c] (5 mM)	−	−	−	−
Freeze-thaw[d]	−	23	−	6
Ether (50%, vol/vol)	4	80	10	84
Toluene (5%, vol/vol)	3	80	7	84

[a] Bacteria were subjected to various treatments in growth medium at a cell density of 4×10^7 CFU per ml. All treatments were at 37°C for 10 min except for ether and toluene extractions (10 s, 4°C) and freeze-thaw. Organic solvents were removed by repeated washings with medium; all other substances were present throughout the incubation (1 h at 37°C). After addition of the lysin (1.5 U/ml) or an equal volume of 50 mM dithiothreitol for control samples, growth or lysis was evaluated after 0, 0.5, and 1 h by measuring the turbidity at 550 nm in a Zeiss spectrophotometer.

[b] —, Bacterial growth (i.e., turbidity greater than at time zero).

[c] Ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid.

[d] Cultures were frozen by immersion in a Dry Ice-acetone bath (−40°C) and thawed immediately at 37°C. This procedure was repeated five times.

member, however, that the cell walls used in these assays contained the radioactive isotope in the biosynthetically old portion of the cell wall. A possible hypersensitivity of murein synthesized during penicillin treatment can not be ruled out by these experiments.

The basis for the resistance of normally growing cells to the lysin is not due to a lack in the physical association between the enzyme and the cells. The lysin could be adsorbed to the surface of live cells in growth medium; the bacteria were then harvested and washed several times. After resuspension in fresh medium, the cells could be lysed by the addition of penicillin (Fig. 6).

Potential inhibitors of the lysin. A possible basis for sensitization could be penicillin-induced release of an inhibitor of the lysin. Supernatant fluids from cultures exposed to penicillin have been tested with little success for lysin-inhibitory activity. We also tested compounds known to be released from the bacteria (LTA, lipids) during penicillin treatment. These compounds were tested in the standard in vitro enzyme assay with a subsaturating amount of lysin but were found to be relatively ineffective; 50% inhibition of lysin by either the unfractionated lipids or by LTA required substantial quantities (1.5 mg/ml). The inhibitory activity may have been suppressed by the presence of EDTA in the buffer (a sensitizing agent in vivo [Table 2] but not essential for lysis in vitro [2]). Therefore the tests were repeated, omitting EDTA (the relative activity of the enzyme was reduced twofold). This increased the LTA-mediated inhibition of the lysin (80% inhibition by 1 mg of LTA per ml); however, the quantities required were still rather large (80% inhibition required 100-fold excess of LTA over the amount of cell wall on a dry weight basis) (Fig. 7).

Sensitization of *S. sanguis* to human lysozyme in vivo. The following experiment was performed to determine whether treatment with penicillin in an analogous fashion could sensitize the organism to human lysozyme. Figure 8 illustrates the results of such an experiment with human urinary lysozyme. This enzyme had less marked effects on penicillin-treated *S. sanguis*; however, a degree of lysis was discernible in cultures exposed simultaneously to penicillin and lysozyme. Contrary to expectations, no killing was detected in these same cultures during the 4-h time period, although a 10-fold-lower survival rate was obtained after 20 h compared with cultures plus penicillin alone (data not shown). This failure to detect killing by lysozyme and penicillin appeared to be related to the dechaining effect of the enzyme. The CFU in cultures exposed to lysozyme were consistently twofold higher than in control cultures; if these same cultures were subjected to vigorous stirring as described in Materials and Methods before plating, this difference in CFU was no longer evident. When cells which had been exposed to penicillin plus or minus lysozyme were subjected to the same mechanical treatment be-

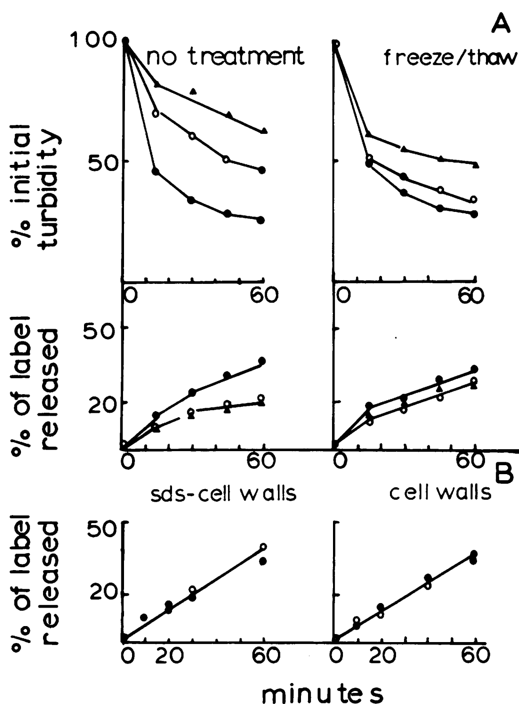

FIG. 5. *Effect of lysin on whole cells (A) and cell walls (B) of S. sanguis. (A) Bacteria which were prelabeled with [^{3}H]GlcNAc and then treated with either chloramphenicol (25 μg/ml) or benzylpenicillin (1 μg/ml; pen) for 30 min (see the text) were resuspended in the original volume of lysis buffer (prewarmed at 37°C). C-phage-associated lysin (0.75 U/ml) was added to each sample, and incubation was continued. Lysis was monitored by measuring the turbidity at 550 nm. The lower portion of the graph indicates the release of radioactivity into the supernatant fluids of the same samples. Symbols:* (O) *control cells;* (●) *penicillin-treated cells;* (▲) *chloramphenicol-treated cells. The left side of the graph illustrates the lysis of untreated cells; the right side illustrates lysis of cells subjected to five cycles of freezing and thawing (see legend to Table 2). (B) Control and penicillin-treated [^{3}H]GlcNAc-labeled bacteria were prepared as described above and combined with identically treated carrier cells for the isolation of cell walls (see the text). Cell walls from 15-ml equivalents of the original cultures were suspended in 1 ml of lysis buffer and exposed to 2 U of lysin per ml (limiting). Solubilization of the radioactivity was determined as described in the text. Symbols:* (O) *cell walls isolated from control culture;* (●) *from penicillin-treated culture. The cell walls on the right-hand side were a crude preparation (isolated from disrupted cells by differential centrifugation and treated with deoxyribonuclease and ribonuclease). Those on the left side were trypsinized, then heated in 2% sodium dodecyl sulfate as described in the text.*

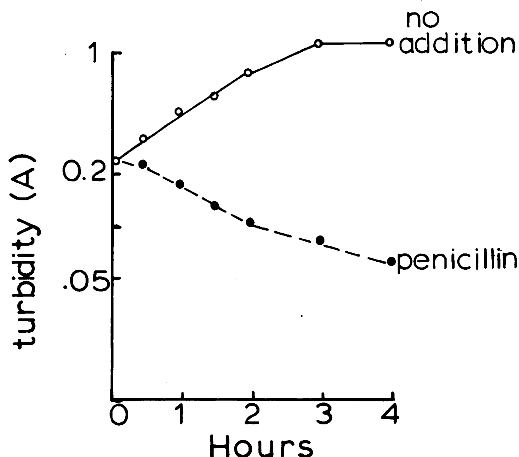

FIG. 6. *Adsorption of lysin to untreated bacteria and subsequent induction of lysis with benzylpenicillin. Bacteria at about 4 × 10^7 CFU per ml were exposed to the lysin (5 U/ml) in 4 ml of C+Y medium for 5 min at 37°C and then for 15 min at 4°C, collected on a membrane filter (0.45 μm), and washed twice with the same volume of medium. After resuspension in 4 ml of fresh prewarmed medium, the culture was divided in half. One half was treated with benzylpenicillin (100 × MIC), and the other served as a control. The samples were incubated for a total of 4 h at 37°C, and growth and lysis were measured by determining the turbidity at 550 nm at intervals. Symbols:* (O) *control cells;* (●) *penicillin-treated cells.*

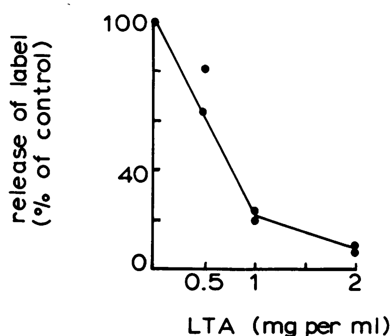

FIG. 7. *Inhibition of the lysin by LTA isolated from the Wicky strain. Enzyme activity was measured in the standard assay (except that EDTA was omitted from the buffer) with [^{3}H]GlcNAc-labeled cell walls as substrate. LTA at concentrations indicated was preincubated with the cell walls for 5 min at 4°C. Ten microliters of lysin was then added, and the mixture was kept at 4°C for an additional 5 min before it was shifted to 37°C and incubated for 30 min. Approximately 50% of the total label was released in the control samples. Release in the presence of LTA is expressed as percentage of this amount.*

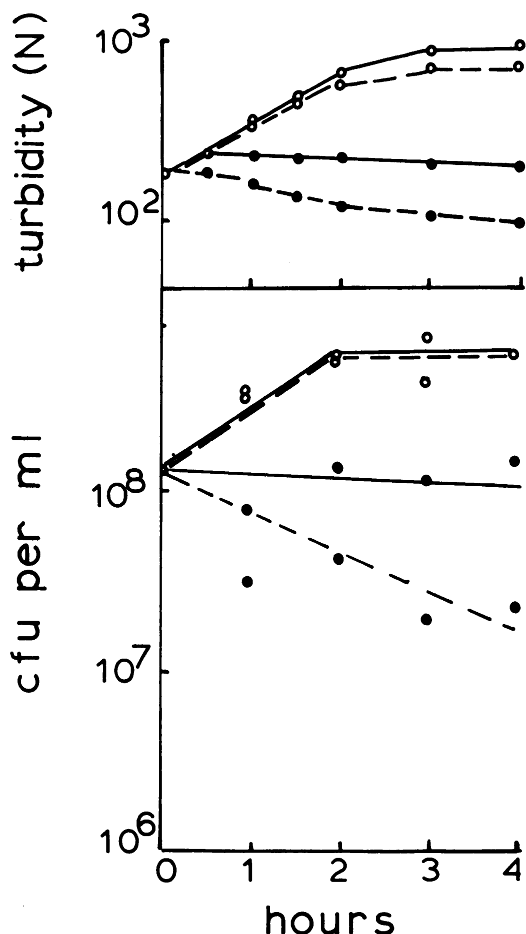

FIG. 8. *Bacteriolytic and bactericidal effects of benzylpenicillin treatment of S. sanguis strain Wicky in the presence of human lysozyme. Ten-milliliter samples of an exponentially growing culture in C+Y medium received penicillin (10 μg/ml) followed by 60 μg of lysozyme per ml. Symbols:* (O) *no antibiotic;* (●) *plus penicillin;* (—) *no lysozyme;* (- - -) *plus lysozyme. Growth, lysis, and viable titer were measured as described in the legend to Fig. 1 except that 1-ml samples were stirred for 2 × 2 min on a tissue homogenizer before plating in order to dechain the bacteria.*

fore plating, a definite decline in viability was apparent only in the sample which had received lysozyme (Fig. 8).

DISCUSSION

Normally growing cells of S. sanguis were found to be resistant to the action of an exogenous murein hydrolase, the C-phage-associated lysin. As indicated here, these bacteria were also tolerant to benzylpenicillin and other cell wall inhibitors (17). However, in seven of the nine strains tested, the simultaneous addition of lysin and a cell wall inhibitor sensitized the cells to the action of the enzyme, eventually resulting in lysis and cell death. Since antibiotics such as rifampin, chloramphenicol, and cerulenin (which act via entirely different mechanisms) were ineffective sensitizing agents, the susceptibility of the cells to the enzyme was not caused solely by inhibition of growth but was somehow related to inhibition of murein biosynthesis. Chloramphenicol or cerulenin in fact antagonized the lethal effect of benzylpenicillin plus enzyme. In many respects, therefore, the phenomenon described here (referred to from now on as "sensitization") is reminiscent of the typical response of wild-type lysis-prone bacteria to penicillin (13, 21, 26, 30). Additionally, both the degree of autolysis and the effectiveness of sensitization to exogenous lysin are proportional to the concentration of the antibiotic; increasing the concentration of the drug increases the rate of lysis and death and decreases the time at which these are apparent (29).

Further exploratory experiments were done to probe the mechanism of this sensitization and its other possible similarities to drug-induced autolysis. It was found that purified cell walls from penicillin-pretreated and from normal cells were lysed at identical rates by an equal quantity of lysin, indicating that penicillin did not cause a gross alteration in murein structure, making it a better substrate for the lysin. However, a localized change in murein structure, perhaps in the growth zone, cannot be excluded at present.

Diverse treatments, including mechanical disruption of growing cells, freezing and thawing, brief toluene or ether extraction, and addition of EDTA, were all found to make cells susceptible to lysin in growth medium. It is of interest that all of these treatments would be expected to act on the cytoplasmic membrane rather than on the cell wall. In contrast, the primary target of the sensitizing antibiotics is known to be the cell wall. Therefore, sensitization by physical and chemical treatments versus by antibiotics may have completely different mechanisms.

On the other hand, treatment of S. sanguis with any effective cell wall inhibitor (whether "early" or "late" inhibitor) induced massive secretion of membrane components (i.e., LTA, lipid; 16, 18). Furthermore, simultaneous inhibition of protein and cell wall biosynthesis reduces secretion of membrane components. Interestingly, these are the very treatments which antagonize the sensitizing effect of cell wall inhibitors. In view of these facts, it seems possible

that the mechanisms of sensitization by antibiotics and by the physical and chemical treatments may have a common basis. We propose as a working hypothesis that sensitization to exogenous murein hydrolases is caused by loss of protective factors that in normally growing bacteria block the lysin (or lysozyme)-sensitive sites within the murein structure. Our model assumes that the width of the murein is penetrated or interdigitated by nonmurein material (which could possibly include LTA, lipid, or other membrane components) that functions as an effective blocking agent. However, based on evidence presented here (see Fig. 6), such hypothetical blocking components do not appear to prevent attachment of lysin to the surface of normally growing bacteria. Since a variety of conditions (i.e., mechanical disruption, organic solvents, and other reagents) render *S. sanguis* cells susceptible to the lysin but do not necessarily induce secretion to membrane components (data not shown; 16, 17), we suggest that sensitizing conditions may cause removal of blocking agents either by physical loss to medium (16–18) or by disruption of a topographical arrangement of blocking factors and cell wall essential for the protection of the murein.

The above model is similar to a previous model proposed to explain the autolysin-triggering effect of cell wall inhibitors on pneumococci (31). In that bacterium, autolysin-defective mutants can be made susceptible to wild-type autolysin during treatment with those antibiotics presumably because these drugs also cause secretion of a specific autolysin inhibitor (the Forssman antigen or lipid-teichoic acid) among other surface components. As in the secretion phenomenon described above, penicillin-induced release of this autolysin inhibitor as well as sensitization to homologous wild-type enzyme were antagonized by inhibition of protein synthesis (31). Later investigations have implicated LTA and phospholipids (i.e., cardiolipin) of gram-positive bacteria as important in the negative control of autolysin activity (3, 4, 15). Similar negative control of autolysins has been proposed in *Bacillus subtilis* by Glaser and Lindsay (12). In this organism, the autolytic enzymes catalyze cell wall turnover but are prevented from lysing the cells. The authors hypothesized the existence of murein-protective material that penetrates a critical width of the cell wall (12).

The location of LTA molecules in the plasma membrane (19) and their massive release from gram-positive bacteria during inhibition of murein synthesis (17, 18) make them plausible candidates as elements which protect the murein in normally growing cells from enzymatic attack by either endogenous or exogenous lytic enzymes. In the case reported here, significant inhibition of the C-phage lysin in an in vitro assay required extremely high concentrations of LTA. However, these negative results may represent technical difficulties in attempting to reconstruct the topography which exists in vivo with these amphipatic molecules.

The endogenous murein hydrolases of gram-negative *Escherichia coli* can be triggered by certain beta-lactam antibiotics and a variety of other treatments (EDTA, trichloroacetic acid, etc.) of whole cells. This triggering has been interpreted in terms of damage to an anatomical barrier (i.e., cytoplasmic membrane) presumed to separate endogenous enzymes from their substrate in living bacteria (14). The latter idea is analogous but not identical to our proposed mechanism for sensitization of *S. sanguis* to the lysin since, in our case, the enzyme attacks from the outside.

Our basic finding is that a normally lysis-defective, penicillin-tolerant bacterium can be made to respond to penicillin in a manner typical of susceptible, highly autolytic bacteria by the simple addition of a heterologous lytic enzyme to the growth medium. Conversely, many treatments (i.e., detergents) which trigger autolysin in lytic bacteria also sensitize *S. sanguis* to externally added enzyme. Therefore, this system may be useful as a model in further experimentation on the mechanism of action of beta-lactams and other cell wall inhibitors. It also lends support to a previous hypothesis that the tolerant response of this organism is due to a natural deficiency in autolysin (17) and further supports the notion that lytic enzymes may play a central role in mediating the irreversible effects of cell wall inhibitors (26, 29).

The penicillin-induced sensitization of *S. sanguis* to human (urinary) lysozyme raises the possibility that the penicillin may act synergistically with host factors during the course of an infection. This speculation is a particularly intriguing one in the case of streptococcal endocarditis, since the cure of endocarditis is usually assumed to require chemotherapy with an effective bactericidal antibiotic (27). Each one of the five clinical isolates (originating from patients with postoperative endocarditis) exhibits various degrees of penicillin tolerance in the experiments described here, indicating that for these bacteria in test tube cultures, penicillin is not an effective bactericidal agent. Yet, medical records indicate that each of the patients was effectively cured by benzylpenicillin chemotherapy. It is conceivable that in vivo, within the infected host, the penicillin treatment sensitizes the pathogens to some host factors, perhaps in a manner analogous to the in vitro sensitization effect de-

scribed here. This synergistic action, rather than the chemotherapeutic agent on its own, would then be responsible for the in vivo elimination of these penicillin-tolerant bacteria.

The idea that penicillin may increase susceptibility of the bacteria to host defense mechanisms is not a new one. Particularly important are the pioneering investigations of Warren and his colleagues, who have already documented a number of cases in which treatment of staphylococci with nafcillin made these bacteria susceptible to exogenous lytic enzymes (32) and to phagocytes (10). Friedman and Warren reported that cyclocillin treatment of *E. coli* has sensitized these bacteria to the antibody-mediated bactericidal effect of complement (11). Additional reports indicate that penicillin pretreatment can increase the susceptibility of staphylococci to leukocyte factors (7) and to phagocytosis (R. D. Root, R. E. Isturitz, and J. A. Metcalf, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, A5, p. 1).

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LITERATURE CITED

1. **Ames, G. F.** 1968. Lipids of *Salmonella typhimurium* and *Escherichia coli*: structure and metabolism. J. Bacteriol. **95**:833-843.
2. **Calandra, G. B., K. M. Nugent, and R. M. Cole.** 1974. Preparation of protoplasts of group H streptococci. Appl. Microbiol. **29**:90-93.
3. **Cleveland, R. F., J. V. Holtje, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and G. D. Shockman.** 1975. Inhibition of bacterial wall lysin by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. **67**:1128-1135.
4. **Cleveland, R. F., A. J. Wicken, L. Daneo-Moore, and G. D. Shockman.** 1976. Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipids. J. Bacteriol. **126**:192-197.
5. **Cole, R. M., G. B. Calandra, E. Huff, and K. M. Nugent.** 1970. Attributes of potential utility in differentiating among "Group H" streptococci and *Streptococcus sanguis*. J. Dent. Res. **55**:A142-A153.
6. **Colman, G., and R. E. O. Williams.** 1972. Taxonomy of human viridans streptococci, p. 281-299. *In* L. S. Wannamaker and J. M. Hatsen (ed.), Streptococci and streptococcal diseases. Academic Press Inc., New York.
7. **Efrati, C., T. Sacks, N. Ne'eman, M. Lahav, and I. Ginsburg.** 1976. The effect of leucocyte hydrolases on bacteria. VII. The combined effect of leukocyte extracts, lysozyme, enzyme "cocktails", and penicillin on the lysis of Staphylococcus aureus and group A streptococci *in vitro*. Inflammation 1:371-407.
8. **Elliot, S. D., M. McCarty, and R. C. Lancefield.** 1977. Teichoic acids of group D streptococci with special reference to strains from pig meningitis (*Streptococcus suis*). J. Exp. Med. **145**:490-499.
9. **Fischetti, V. A., E. C. Gotschlich, and A. W. Bernheimer.** 1971. Purification and physical properties of group C streptococcal phage-associated lysin. J. Exp. Med. **114**:1105-1117.
10. **Friedman, H., and G. H. Warren.** 1974. Enhanced susceptibility of penicillin-resistant staphylococci to phagocytosis after *in vitro* incubation with low doses of nafcillin. Proc. Soc. Exp. Biol. Med. **146**:707-711.
11. **Friedman, H., and G.H. Warren.** 1976. Antibody-mediated bacteriolysis: enhanced killing of cyclocillin treated bacteria. Proc. Soc. Exp. Biol. Med. **153**:301-304.
12. **Glaser, L., and B. Lindsay.** 1977. Relation between cell wall turnover and cell growth in *Bacillus subtilis*. J. Bacteriol. 130:610-619.
13. **Goodell, E. W., R. Lopez, and A. Tomasz.** 1976. Suppression of lytic effect of beta lactams on *Escherichia coli* and other bacteria. Proc. Natl. Acad. Sci. U.S.A. **73**:3293-3297.
14. **Hartman, R., S. B. Bock-Hennig, and U. Schwarz.** 1974. Murein hydrolases in the envelope of *Escherichia coli*. Properties *in situ* and solubilization from the envelope. Eur. J. Biochem. **41**:203-208.
15. **Höltje, J. W., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. **72**:1690-1694.
16. **Horne, D., R. Hakenbeck, and A. Tomasz.** 1977. Secretion of lipids induced by inhibition of peptiglycan synthesis in streptococci. J. Bacteriol. **132**:704-717.
17. **Horne, D., and A. Tomasz.** 1977. Tolerant response of *Streptococcus sanguis* to beta lactams and other cell wall inhibitors. Antimicrob. Agents Chemother. **11**: 888-896.
18. **Horne, D., and A. Tomasz.** 1979. Release of lipoteichoic acid from *Streptococcus sanguis*: stimulation of release during penicillin treatment. J. Bacteriol. **137**:1180-1184.
19. **Knox, K. W., and A. J. Wicken.** 1970. Immunologic properties of teichoic acids. Bacteriol. Rev. **37**:219-257.
20. **Lacks, S., and R. D. Hotchkiss.** 1960. A study of the genetic material determining an enzyme activity in Pneumococcus. Biochim. Biophys. Acta **39**:508-517.
21. **Lopez, R., C. Ronda-Lain, A. Tapia, S. B. Waks, and A. Tomasz.** 1976. Suppression of the lytic and bactericidal effects of cell wall-inhibitory antibiotics. Antimicrob. Agents Chemother. **10**:697-706.
22. **Mosser, J. S., and A. Tomasz.** 1970. Choline containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **245**:287-298.
23. **Ofek, I., E. H. Beachey, W. Jefferson, and G. L. Campbell.** 1975. Cell membrane-binding properties of group A streptococcal lipoteichoic acid. J. Exp. Med. **141**:990-1003.
24. **Omura, S.** 1976. The antibiotic cerulenin, an novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40**:681-697.
25. **Rantz, L. A., and E. Randall.** 1955. Use of autoclaved extracts of haemolytic streptococci for serological grouping. Stanford Med. Bull. **13**:290-291.
26. **Rogers, H. J., and C. W. Forsberg.** 1971. Role of autolysins in killing of bacteria by some bactericidal antibiotics. J. Bacteriol. **108**:1235-1243.
27. **Sabath, L. D.** 1979. Staphylococcal tolerance to penicillins and cephalosporins, p. 299-303. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
28. **Tomasz, A.** 1970. Cellular metabolism in genetic transformation of pneumococci: requirement for protein synthesis during induction of competence. J. Bacteriol. **101**:860-871.

29. **Tomasz, A.** 1974. The role of autolysins in cell death. Ann. N.Y. Acad. Sci. **235**:439–447.
30. **Tomasz, A., A. Albino, and E. Zanati.** 1970. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature (London) **227**:138–140.
31. **Tomasz, A., and S. Waks.** 1975. Mechanism of action of penicillin: triggering of the pneumococcal autolytic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. **72**:4162–4166.
32. **Warren, G. H., and J. Gray.** 1967. Influence of nafcillin on the enzymatic lysis of *Staphylococcus aureus*. Can. J. Microbiol. **13**:321–328.