INFECTION AND IMMUNITY, May 1982, p. 582–585
0019-9567/82/050582-04$02.00/0

Vol. 36, No. 2

Deficient Autolytic Enzyme Activity in Antibiotic-Tolerant Lactobacilli

KWANG S. KIM,[1]* JOAN O. MORRISON,[2] AND ARNOLD S. BAYER[2]

Departments of Pediatrics[1] and Medicine,[2] Harbor-University of California at Los Angeles Medical Center, Torrance, California 90509, and the University of California at Los Angeles School of Medicine, Los Angeles, California 90024

Received 14 August 1981/Accepted 17 December 1981

To define the mechanism(s) of penicillin tolerance in lactobacilli, one nontolerant and two tolerant strains were examined for autolytic enzyme activity. When incubated with ^{14}C-labeled cell wall preparations, autolysin extracts of tolerant lactobacilli released significantly less radioactivity than did extracts of nontolerant lactobacilli ($p < 0.02$). These differences in the release of radioactivity by nontolerant and tolerant strains were maximal during the logarithmic growth phase. Moreover, this activity was greatest at pH 8, was heat labile, and was inhibited by the addition of magnesium, suggesting characteristics of an enzyme. This study illustrates that autolytic enzyme activity is deficient in strains of antibiotic-tolerant lactobacilli and suggests that this may be partially responsible for the delayed killing effect of penicillins against such strains.

Serious *Lactobacillus* infections, particularly endocarditis, can result in therapeutic failure despite treatment with appropriate single-drug regimens and the achievement of serum antibiotic concentrations which exceed minimal inhibitory concentrations (1). The reasons for these poor responses in *Lactobacillus* infections are undoubtedly complex, but one proposed explanation is antibiotic tolerance, defined as diminished killing by growth-inhibiting antibiotic concentrations (10). Previous in vitro studies have revealed that most clinical *Lactobacillus* isolates exhibit minimal bactericidal concentrations for cell wall-active antibiotics that are 30- to 200-fold greater than the corresponding minimal inhibitory concentrations. Also, these isolates are not killed by such antibiotics at clinically achievable serum concentrations (2, 4), thus fulfilling in vitro criteria for antibiotic tolerance. In this report, we studied the relationship between penicillin tolerance in lactobacilli and autolytic enzyme activity, in an in vitro radioisotopic assay system.

(This work was presented in part at the 20th Interscience Conference on Antimicrobial Agents and Chemotherapy, 24 Sept. 1980, New Orleans, La.)

MATERIALS AND METHODS

Organisms. One nontolerant (*L. plantarum*) and two tolerant lactobacilli (*L. casei* and *L. plantarum*) were used for this study. These were clinical isolates recovered from blood and were identified and speciated according to Bergey's criteria (9) and by the method of Holdeman and Moore (7). The strains were maintained on brain heart infusion agar slant (BBL Microbiology Systems, Cockeysville, Md.) at 4°C. Two days before each experiment, organisms were transferred to fresh tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 37°C. These 24-h cultures were then inoculated in a 1:00 dilution into fresh tryptose phosphate broth and incubated aerobically at 37°C. Growth curves for tolerant and nontolerant lactobacilli were constructed by determining the numbers of colony-forming units at time zero and at 2, 6, 12, 24, and 48 h of incubation (2).

The determination of penicillin tolerance or nontolerance and the related clinical information for each strain have been previously reported (1, 4). Briefly, penicillin tolerance was determined by the timed killing assays in the presence of penicillin G concentrations of 10 μg/ml (4). When inocula of $\sim 10^5$ colony-forming units per ml were added to the penicillin-containing tubes, a slow bactericidal effect was seen against tolerant strains over 24 to 48 h, with <90% killing of the original inoculum. In contrast, base-line colony-forming units of the nontolerant strains were rapidly reduced >99.9% at 24 h of incubation in penicillin-containing tubes.

Autolysin assay. Crude autolysin preparations were extracted from logarithmic- and stationary-phase cultures of nontolerant and tolerant lactobacilli by a freeze-thaw procedure (8). Lactobacillus cultures in the above-mentioned growth phases were harvested by centrifugation (8,000 × *g* for 20 min), and the cell pellet was washed twice with 0.05 M Tris buffer (pH 8.0). The cells were then suspended in Tris buffer in 10% of the culture volume and frozen at −20°C overnight. Extracts containing autolytic enzyme activity were obtained by thawing the cells at room temperature and centrifugation at 12,000 × *g* for 10 min. The supernatant was concentrated 10-fold (by volume) with an ultrafiltration membrane (Diaflow PM 10,

Amicon, Lexington, Mass.). The protein content in freeze-thaw extracts was measured by the Biorad protein assay (Bio-Rad Laboratories, Richmond, Calif.). The extracts were stored at −20°C at a protein concentration of ~100 μg/ml and tested within 2 days.

Substrates for the autolysin assay were prepared from an *L. casei* isolate grown overnight in tryptose phosphate broth containing 0.1 μCi of L-[*U*-^{14}C]lysine monohydrochloride (341 μCi/mmol; Amersham, Arlington Heights, Ill.) per ml and harvested by centrifugation. The cell pellet was next washed three times with sterile distilled water and suspended in 10% trichloroacetic acid. The suspension was then incubated at 95°C for 10 min and centrifuged at 4,000 × *g* for 10 min. The sediment was washed five to six times with distilled water (sufficient to reduce the counts released from ^{14}C-labeled cell walls to <50 cpm/50 μl in the supernatant). The sediment usually contained 2 × 10^4 to 3 × 10^4 cpm/mg (dry weight). The [^{14}C]lysine-labeled substrate was resuspended in Tris buffer in 1% of the culture volume and was stored at −20°C in samples until used for the assay. The resulting suspension usually contained 4 × 10^4 to 6 × 10^4 cpm/ml.

The assay for autolytic enzyme activity was modified from the method of Best et al. (5). The assay is based on the release of radioactivity from ^{14}C-labeled substrate upon the addition of autolysin extracts of nontolerant and tolerant lactobacilli. In the current experiment, the reaction mixture contained 0.6 ml of radiolabeled substrates (~1.2 mg by dry weight), 0.8 ml of freeze-thaw (autolysin) extracts from either nontolerant or tolerant strains (~80 μg of protein), and 0.1 ml of Tris buffer or 10^{-2} M $MgCl_2$. Control tubes with Tris buffer instead of autolytic extracts and extracts preheated at 100°C for 10 min were also included. In addition, the autolytic assays were repeated at pH 6, 7, 8, and 9.

Thawed extracts were added to the radiolabeled substrate, and 0.2-ml samples were removed at time zero and at 1, 2, 3, and 16 h; these samples were then added to 0.1 ml of chilled water and centrifuged immediately (8,000 × *g* for 10 min). A total of 50 μl of the supernatant was then transferred to counting vials containing 10 ml of Ready-Solv Hp (Beckman Instruments, Fullerton, Calif.), and radioactivity was counted in a Mark II scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). A separate substrate preparation was used for each experiment. Autolytic enzyme activity was expressed as the percentage of counts per minute released during the 16-h incubation from the total radioactivity of the cell wall substrate at time zero.

Statistical methods. Differences in autolytic enzyme activities between tolerant and nontolerant lactobacilli were analyzed by the Student *t* test (6).

RESULTS

Figure 1 shows the effect of bacterial growth phase on autolytic enzyme activity. The growth curves for nontolerant and tolerant strains were virtually identical, reaching the late-logarithmic phase at 12 h and stationary phases after 24 h of incubation. For nontolerant and tolerant lactobacilli, autolytic enzyme activity was greatest in the late-logarithmic phase and least in the stationary phase of growth. However, at each point of different growth phases, autolytic enzyme activities of a nontolerant strain were greater than those of tolerant strains. Table 1 shows the results of a representative experiment. At 1, 2, 3 and 16 h, the autolytic enzyme activities of a nontolerant strain were significantly greater than those of a tolerant strain ($P < 0.05$). The amount of [^{14}C]lysine released by a tolerant strain during the first 3 h of incubation was negligible and equivalent to the buffer effect (≤110 cpm/50 μl of the supernatant and ≤14% of the total radioactivity of the substrate in this experiment). When the autolysin assay was performed at pH 6 to 9, the release of [^{14}C]lysine was greatest at

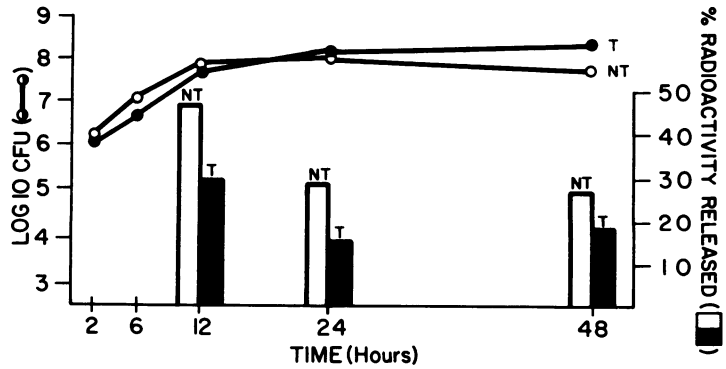

FIG. 1. Effect of bacterial growth phase on autolytic enzyme activity. The growth curves for nontolerant (NT) and tolerant (T) lactobacilli are shown as a solid line. Autolytic enzyme activities were expressed as the percentage of counts per minute released during 16 h of incubation from the total radioactivity of ^{14}C-labeled substrate by autolytic enzyme extracts of nontolerant and tolerant lactobacilli. The autolytic enzyme activities of nontolerant and tolerant strains at 12, 24, and 48 h of growth are shown as a bar. Data from the other tolerant strain were nearly identical to those shown for the tolerant strain depicted here.

TABLE 1. Results of a representative experiment comparing autolytic enzyme activities between a nontolerant and a tolerant *Lactobacillus* strain[a]

Source of freeze-thaw extracts	Radioactivity in the supernatant (cpm/50 μl)				
	0 h	1 h	2 h	3 h	16 h
Nontolerant	87 (11%)[b]	165 (21%)	203 (26%)	207 (26%)	285 (36%)
Tolerant	87 (11%)	79 (10%)	89 (11%)	97 (12%)	185 (23%)
Buffer	87 (11%)	93 (12%)	110 (14%)	81 (10%)	104 (13%)

[a] Autolytic enzyme activities were expressed as actual counts per minute released into the supernatant (50 μl), from [^{14}C]lysine-labeled cell wall substrate (4.3×10^4 cpm/ml) at time zero and at 1, 2, 3, and 16 h, by equivalent protein amounts (~80 μg) of freeze-thaw extracts of a nontolerant and a tolerant strain. Tris buffer instead of freeze-thaw extracts was used as a control.

[b] The percentages in parentheses represent the proportions of the total radioactivity contained in the substrate, released at various times of incubation.

pH 8 and least at pH 6, suggesting that this activity is pH dependent (data not shown). Moreover, when the extracts of nontolerant strains were heated, autolytic enzyme activity was decreased to the level of tolerant strains or buffer, indicating that this activity was heat labile. Also, when 10^{-2} M $MgCl_2$ was added, the activity of a nontolerant strain was decreased to the level of a tolerant strain, and this difference was significant ($P < 0.01$).

DISCUSSION

Clinically, lactobacillemia associated with deep-seated foci, particularly endocarditis, has been relatively refractory to high-dose parenteral therapy with the penicillins, despite readily attainable mean inhibitory concentrations in serum (1). Our previous studies have suggested that the discrepancy between the activity in vitro and suboptimal therapeutic responses in vivo may be related to antibiotic tolerance, defined in vitro as delayed or diminished killing by the growth inhibitory concentration of the antibiotic (2, 4). In addition, this slow bactericidal effect of β-lactam agents in vitro is paralleled in vivo in the experimental rabbit model of antibiotic-tolerant *L. plantarum* endocarditis (3).

In this report, we studied the mechanism of antibiotic tolerance in lactobacilli in terms of autolytic enzyme activity and found several significant findings: (i) when incubated with ^{14}C-labeled cell wall substrate, freeze-thaw extracts of tolerant lactobacilli released significantly less radioactivity than did extracts of nontolerant strains; and (ii) the relative autolytic enzyme activity of freeze-thaw extracts from nontolerant and tolerant strains was dependent on the growth phase of the organism. Significantly more autolytic activity was observed in extracts from logarithmic-phase cultures than stationary-phase cultures; (iii) autolytic enzyme activity of a nontolerant strain was reduced to levels seen in tolerant strains or buffer by heat treatment, and additions of Mg^{2+}, suggesting an enzymatic property (5, 10).

In this study, we did not attempt to purify relatively crude freeze-thaw extracts or [^{14}C]lysine-labeled substrate. However, the differences in radioactivity released by nontolerant and tolerant lactobacilli were significant ($P < 0.05$). It appears that penicillin tolerance in lactobacilli is related to a defective autolytic enzyme system. Further investigations are needed to differentiate between an absolute decrease in autolytic enzyme levels or increased autolytic inhibitor activity as an explanation of diminished autolytic activity seen in tolerant lactobacilli.

ACKNOWLEDGMENTS

This work was supported in part by Institutional National Research Service award AI 07014 and research grant AI 15698 from the National Institute of Allergy and Infectious Diseases, and research grant-in-aid 541 C-2 from the American Heart Association-Los Angeles Chapter.

The authors thank Bascom F. Anthony for his review of the manuscript and for his comments.

LITERATURE CITED

1. **Bayer, A. S., A. W. Chow, D. Betts, and L. B. Guze.** 1978. Lactobacillemia—report of nine cases. Am. J. Med. **64**:808–813.
2. **Bayer, A. S., A. W. Chow, N. Concepcion, and L. B. Guze.** 1978. Susceptibility of 40 lactobacilli to six antimicrobial agents with broad gram-positive anaerobic spectra. Antimicrob. Agents Chemother. **14**:720–722.
3. **Bayer, A. S., A. W. Chow, K. Ishida, J. O. Morrison, and L. B. Guze.** 1981. Therapy of experimental endocarditis due to antibiotic-tolerant *Lactobacillus plantarum*—bactericidal synergy of penicillin plus gentamicin. Correlation of *in vitro* susceptibility studies with *in vivo* efficacy. Chemotherapy **27**:444–451.
4. **Bayer, A. S., A. W. Chow, J. O. Morrison, and L. B. Guze.** 1980. Bactericidal synergy between penicillin or ampicillin and aminoglycosides against antibiotic-tolerant lactobacilli. Antimicrob. Agents Chemother. **17**:359–363.
5. **Best, G. K., N. H. Best, and A. V. Koval.** 1974. Evidence for participation of autolysins in bactericidal action of oxacillin on *Staphylococcus aureus*. Antimicrob. Agents Chemother. **6**:825–830.
6. **Colton, T.** 1974. Statistics in medicine, p. 99–150. Little, Brown & Co., Boston.
7. **Holdeman, L. V., and W. E. C. Moore (ed).** 1973. Anaerobic laboratory manual. Virginia Polytechnic Institute and

State University, Blacksburg, Va.
8. **Huff, E., and C. S. Silverman.** 1968. Lysis of *Staphylococcus aureus* cell walls by a soluble staphylococcal enzyme. J. Bacteriol. **95**:99–106.
9. **Rogosa, M.** 1974. Gram-positive, asporogenous, rod-shaped bacteria, p. 576–593. *In* R. E. Buchanan and N. E. Gibbons (ed)., Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore, Md.
10. **Sabath, L. D., N. Wheeler, M. Laverdiere, D. Blazevic, and B. J. Wilkinson.** 1977. A new type of penicillin resistance of *Staphylococcus aureus*. Lancet **i**:443–447.