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Can Penicillins and Other β-Lactam Antibiotics Be Used To Treat Tuberculosis?

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An increase in the number of tuberculosis cases caused by multiple-drug-resistant strains of *Mycobacterium tuberculosis* has stimulated search for new antituberculous agents. Beta-lactam antibiotics, traditionally regarded as ineffective against tuberculosis, merit consideration. Four major penicillin-binding proteins (PBPs) with approximate molecular sizes of 94, 82, 52, and 37 kDa were detected by fluorography of [^{3}H]penicillin-radiolabeled membrane proteins prepared from *M. tuberculosis* H37Ra. The presence of membrane-associated β-lactamase precluded the use of membranes for assaying the binding affinities of beta-lactam antibiotics. Therefore, ampicillin affinity chromatography was used to purify these four PBPs from crude membranes in order to assay the binding affinities of beta-lactam antibiotics. Ampicillin, amoxicillin, and imipenem, beta-lactam antibiotics previously reported to be active in vitro against *M. tuberculosis*, bound to *M. tuberculosis* PBPs at therapeutically achievable concentrations. Binding of the 94-, 82-, and 52-kDa PBPs, but not the 37-kDa PBP, was associated with antibacterial activity, suggesting that these PBPs are the critical targets. Studies of mycobacterial cell wall permeability, which was assayed with a panel of reference cephalosporins and penicillins with different charge positivities, indicated that the rate of penetration of beta-lactam antibiotics to the target PBPs could not account for resistance. Resistance could be reversed with the β-lactamase inhibitors clavulanate or sulbactam or could be circumvented by the use of a β-lactamase-stable drug, imipenem, indicating that mycobacterial β-lactamase, probably in conjunction with slow penetration, is a major determinant of *M. tuberculosis* resistance to beta-lactam antibiotics. These findings confirm in vitro data that *M. tuberculosis* is susceptible to some beta-lactam antibiotics. Further evaluation of these drugs for the treatment of tuberculosis in animal models and in clinical trials is warranted.

Tuberculosis caused by drug-resistant strains of *Mycobacterium tuberculosis* is a growing problem (2, 12, 15). Screening, discovery, and clinical evaluation of novel compounds takes years, but effective agents are needed now. If antituberculous agents could be identified from among the antimicrobial agents that are already available, this would greatly speed drug development. Some beta-lactam antibiotics appear to be active in vitro (6, 8, 29, 31, 33), but since they have not been used to treat tuberculosis, it is uncertain if this is clinically meaningful. Regardless of in vitro susceptibility data, *M. tuberculosis* historically has been regarded as intrinsically resistant to beta-lactam antibiotics (10) because of its β-lactamase that inactivates penicillins and cephalosporins (18, 19) and its lipid-rich outer cell wall that is believed to be a virtually impenetrable barrier to polar molecules such as beta-lactam antibiotics (16, 17, 30).

An absolute requirement for a beta-lactam antibiotic to be an effective antituberculous agent is that it must tightly bind to its target. The targets are penicillin-binding proteins (PBPs), essential membrane-bound cell wall synthetic enzymes (13), which covalently bind to and are irreversibly inhibited by beta-lactam antibiotics. Susceptible bacteria produce PBPs with high affinities for these drugs; i.e., binding occurs at clinically achievable concentrations. If *M. tuberculosis* PBPs have a high affinity, then beta-lactam antibiotics may very well be effective agents. We characterized the binding of several beta-lactam antibiotics to PBPs of *M. tuberculosis* H37Ra and examined the relationship between binding of PBPs and in vitro susceptibility.

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MATERIALS AND METHODS

Bacterial strains. Strain H37Ra (ATCC 25177) is an attenuated laboratory strain of *M. tuberculosis*, and strain H37Rv (ATCC 25618) is its virulent variant. The five clinical strains were sputum isolates obtained from the Clinical Microbiology Laboratories of San Francisco General Hospital.

Preparation of membranes. Mid-exponential-phase cells grown in Middlebrook 7H9 broth supplemented with 5 g of bovine serum albumin, 2 g of glucose, 3 mg of catalase, and 2 g of glycerol were washed once in 50 mM potassium phosphate–1 M NaCl (pH 7.0) buffer at 4°C, concentrated, and shattered with 100-μm-diameter glass beads at 4°C for 5 min in a mechanical cell disintegrator. Membranes were prepared by differential centrifugation (7), suspended in 10 mM potassium phosphate buffer (pH 7.0) at a concentration of 10 mg/ml, and stored at −70°C.

Affinity purification of PBPs. To purify the PBPs, affinity chromatography of mechanically disrupted whole cells was performed with ampicillin coupled to activated CH Sepharose 4B (Pharmacia) (34). The PBPs were eluted with 1 M hydroxylamine in 50 mM potassium phosphate (pH 7.0) buffer, dialyzed against 10 mM potassium phosphate (pH 7.0) buffer, and concentrated by ultrafiltration centrifugation.

PBP binding assay. Ampicillin affinity-purified PBP samples of approximately 5 μg of protein were incubated with antibiotics at final concentrations of 0, 0.1, 0.5, 1, 5, 10, 20, 50, and 100 μg/ml at 37°C for 20 min, and then 20 μg (final concentrations) of [^{3}H]benzylpenicillin (30 Ci/mmol; a gift from Merck Sharpe & Dohme) per ml was added for 20 min. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide, 0.26% bisacrylam-

ide), and PBPs were detected by fluorography (22, 23). The relative density of each PBP at each concentration was measured by scanning densitometry. The 50% inhibitory concentration (IC_{50}) was calculated by linear regression for PBP density as a percentage of that of the control plotted against concentration.

Assay of β-lactamase activity. Residual β-lactamase activity in affinity-purified PBPs was detected by a nitrocephin disk assay (Cefinase; BBL) after 30 to 60 min (27). β-Lactamase activity for inhibition experiments was assayed spectrophotometrically (36). β-Lactamase was prepared from the cell wall fraction of mechanically disrupted cells sonicated in 50 mM potassium phosphate–0.1% Triton X-100 (pH 7.0) buffer. Particulate debris was removed by centrifugation. Supernatant containing 2.5 to 10 μg of protein was added to 100 μM nitrocephin (BBL) in 50 mM potassium phosphate (pH 7.0) buffer plus β-lactamase inhibitor at a final concentration of 0, 0.1, 1, or 10 μg/ml. β-Lactamase activity was expressed as the rate of change in the A_{482}; the IC_{50} was defined as the concentration that reduced that rate by 50%.

Determination of MICs. MICs were determined with a BACTEC TB System (Becton-Dickinson Diagnostic Instrumentation Systems, Sparks, Md.) by standard methods (14, 28). MICs were read on day 5 or 6, when the growth index of the control vial was ≥30 dpm.

Determination of antibiotic half-life. Antibiotic was added at a concentration of 1 mg/ml to BACTEC 12B medium (pH 7.0), and aliquots were stored at −20°C or were incubated at 37°C. MICs for either *Escherichia coli* 25992 or *Staphylococcus aureus* 209P were determined by the broth dilution method in Mueller-Hinton broth on successive days for both the frozen aliquot and the 37°C aliquot. The ratios of these two MICs was determined, and the half-life was calculated from the slope of the regression curve obtained by plotting the $\log_2$ ratio versus the number of days of incubation at 37°C.

Assay of mycobacterial cell wall permeability to beta-lactam antibiotics. Cell wall permeability was determined by assaying the entry of beta-lactam antibiotics via spontaneous diffusion based on the rate of hydrolysis by periplasmic *M. tuberculosis* β-lactamase (17, 30). For permeability to be assayed by this spectrophotometric method, the beta-lactam antibiotic must be hydrolyzed at a relatively rapid rate in order to detect the product of hydrolysis or a reduction in substrate concentration (26). The cephalosporins and penicillins used here were hydrolyzed at rates of 0.2 to 0.5 μmol/mg of protein per min when they were assayed with 0.05 to 0.1 mM substrate. A panel of four reference cephalosporins differing in hydrophobicity and four penicillins differing in charge were used. The K_m values for most cephalosporins were 0.1 to 0.2 mM, but those for most penicillins were about 1 mM or higher. Since *M. tuberculosis* cells formed aggregates, the assay was modified as follows. Cells from 400 ml of culture were washed once in 0.05 M Tris-HCl buffer (pH 7.0) and were resuspended in 16 ml of the same buffer. Drugs were added to a 1-mM final concentration at room temperature, and portions of the suspension were filtered through 0.45-μm-pore-size membrane filter disks (Super Acrodisc; Gelman Scientific) at 15-min intervals. The unhydrolyzed cephalosporin remaining in the filtrate was determined from the UV absorption spectra, and the amount of unhydrolyzed penicillin was determined from the decrease in the optical density at 232 nm (for amoxicillin, optical density at 245 nm) upon the addition of purified PSE4 β-lactamase. Correction for hydrolysis caused by β-lactamase that leaked out was unnecessary because this was always less than 1% of the measured hydrolysis rates.

Determination of intracellular elimination of *M. tuberculosis*. J774 cells (derived from mouse peritoneal macrophages) were suspended in Dulbecco's modified Eagle's medium with 5% newborn calf serum (35). After an overnight incubation of the J774 cells at 35°C in 5% CO_2, cells of strain H37Ra were added to each flask to give a 3:1 ratio of H37Ra cells to macrophages. Following 6 h of incubation to allow for macrophage ingestion of the H37Ra cells, the medium was removed from each flask and the contents were washed with 25 ml of Hank's balanced salt solution. A total of 5 ml of fresh medium was added along with the appropriate concentration of antimicrobial agents. The medium was exchanged each day. At time points of 0, 3, 5, and 7 days, medium was removed from the appropriate flask and 5 ml of sterile distilled water was added for 20 min at room temperature. The contents were mixed thoroughly by vigorously pipetting fluid up and down approximately 20 times. Appropriate dilutions of the flask contents were made with sterile deionized water, and 0.1 ml of the dilutions were plated onto Middlebrook 7H10 medium. The plates were incubated at 35°C, and the members of CFU were counted.

RESULTS AND DISCUSSION

Four PBPs with molecular sizes of 94, 82, 52, and 35 kDa were detected in crude membranes (Fig. 1A) radiolabeled with 20 μg of [^{3}H]penicillin per ml. The three higher-molecular-mass PBPs, designated PBPs 1, 2, and 3, respectively, according to decreasing molecular size, but not the 35-kDa PBP 4, were also found to be radiolabeled at a [^{3}H]penicillin concentration of 1 μg/ml. Radiolabeling with 50 μg of [^{3}H]penicillin per ml did not reveal any additional PBPs, but it did significantly increase the background density. The addition of clavulanic acid (to inactive membrane-associated β-lactamase that might hydrolyze the radiolabel, thereby preventing the detection of lower-affinity PBPs that might be present) prior to radiolabeling also failed to reveal other PBPs.

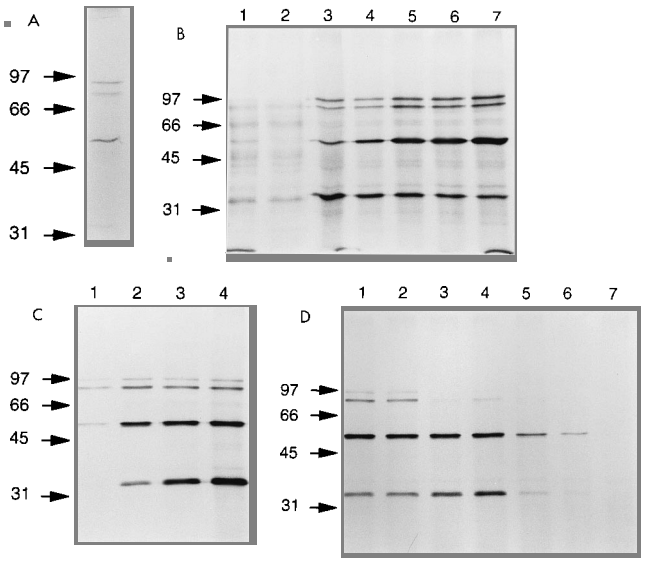

FIG. 1. Fluorographs of PBPs of *M. tuberculosis* H37Ra. Numbers at the left of each panel indicate molecular sizes (in kilodaltons). (A) Membrane fraction radiolabeled with 20 μg of [^{3}H]penicillin per ml. (B) Ampicillin affinity-purified PBPs. Lane 1, disrupted whole cells; lane 2, flowthrough; lanes 3 to 7, successive neutral 1 M hydroxylamine eluates. (C) Direct radiolabeling of ampicillin affinity-purified PBPs with 0.1 (lane 1), 1 (lane 2), 10 (lane 3), and 20 (lane 4) μg of [^{3}H]penicillin per ml. (D) Sulbactam competition assay of affinity-purified PBPs; lanes 1 through 7 0, 0.1, 1, 10, 20, 50, and 100 μg of sulbactam per ml, respectively, added prior to the addition of 20 μg of [^{3}H]penicillin per ml.

The relatively small amount of PBPs that were radiolabeled in crude membrane preparations and the presence of significant amounts of membrane-associated β-lactamase that could interfere with binding assays rendered membrane samples unsuitable for determination of PBP binding affinities for beta-lactam antibiotics. To circumvent these problems, ampicillin affinity chromatography was used to purify sufficient quantities of PBPs from membranes for assays of binding affinity. Substitution of 6-aminopenicillanic (6-APA) or imipenem for ampicillin in the chromatographic procedure produced less satisfactory results, with poor yields of PBPs 1 and 2 with 6-APA and PBP 4 with imipenem. The same four PBPs (i.e., PBPs with the same molecular sizes and radiolabeling characteristics) that were detected in crude membranes were purified by ampicillin affinity chromatography (Fig. 1A and B). As in the membranes, affinity-purified PBPs 1, 2, and 3 radiolabeled at relatively low concentrations of [^{3}H]penicillin: they were detectable at 0.1 μg/ml and were saturated at a concentration of 1 μg/ml (Fig. 1C). PBP 4 was saturated at concentrations of 10 to 20 μg of [^{3}H]penicillin per ml. The specificity of binding was confirmed in competition assays (Fig. 1D) in which increasing amounts of cold beta-lactam antibiotic blocked binding of [^{3}H]penicillin. β-Lactamase activity which copurified with the affinity-purified PBPs was present only in trace amounts that did not significantly affect binding assays. In some experiments PBP 2 and PBP 3 appeared as doublets (data not shown). However, this was not a consistently reproducible result, and the resolution was not sufficient to permit separation of the doublet by scanning densitometry, so for the purposes of the binding assays, PBP 2 and PBP 3 each were considered single protein species. The presence of four major PBPs with apparent molecular masses of 94, 82, 52, and 37 kDa in *M. tubercu-*

TABLE 1. IC_{50}s for PBPs of *M. tuberculosis* H37Ra

Antibiotic	IC_{50} (μg/ml) for PBP of:			
	94 kDa	82 kDa	52 kDa	35 kDa
Amoxicillin	0.2	0.7	0.8	>50
Ampicillin	0.1	1.2	1.2	>50
Nafcillin	0.3	0.3	>10	>10
Clavulanate[a]	0.5	1.3	14	11
Sulbactam[a]	0.4	0.9	>50	>50
Cefoperazone	13	20	2	>50
Cefoxitin	0.2	0.4	2	0.3
Ceftriaxone	0.1	0.1	0.5	>50
Cephaloridine	0.4	1.2	0.6	>50
Cephalothin	2.6	8.3	0.8	>50
Imipenem	0.03	0.1	0.4	0.8

[a] β-Lactamase inhibitor.

losis H37Ra is consistent with the PBPs that have been described in strains of *Mycobacterium avium-Mycobacterium intracellulare* and *Mycobacterium fortuitum*. *M. avium-M. intracellulare* produces three major PBPs (each of which can be resolved into components a and b) with apparent molecular masses 90 to 94, 55 to 60, and 32 to 37 kDa; two minor PBPs (approximately 6% of the total penicillin-binding capacity) of 75 and 44 to 46 kDa were also described (24). *M. fortuitum* produces four major PBPs with apparent molecular masses of 102, 90, 87, and 50 kDa (9).

PBPs 1 and 2 had high binding affinities in competition assays for most beta-lactam antibiotics (IC_{50}s, approximately 1 μg/ml or less), including the β-lactamase inhibitors clavulanic acid and sulbactam (Fig. 1D and Table 1). The affinities of these PBPs for cephalothin and cefoperazone were low. PBP 3 also bound all beta-lactam antibiotics tested except nafcillin and the two β-lactamase inhibitors with a high affinity. The affinity of PBP 4 was low for all drugs except cefoxitin and imipenem.

Cell wall permeability was determined with four reference cephalosporins exhibiting a range of hydrophobicity values and with four penicillins which differed with respect to the degree of charge positivity (ampicillin and amoxicillin > benzylpenicillin > carbenicillin). Hydrophobicity and charge are important determinants of drug penetration through the outer cell membrane of gram-negative bacteria (26). The rates of penetration of these beta-lactam antibiotics though the outer cell wall of *M. tuberculosis* H37Ra ranged between 1.6 to 9.4 nm/s (Table 2). These rates were 10-fold more rapid than those for *Mycobacterium chelonei*, similar to rates for *Pseudomonas aeruginosa*, and about 100-fold less than rates for *E. coli* (26). As has been observed with *E. coli*, cephaloridine penetrated the best, despite its relatively high degree of hydrophobicity. Penetration rates for the other beta-lactams were all about the same, regardless of differences in charge and hydrophobicity. Even with these relatively slow penetration rates compared with those in *E. coli*, half-equilibration across the cell wall is achieved after several minutes (for calculations, see reference 26). In view of the long generation times (18 to 24 h) of *M. tuberculosis*, this is sufficient time for drug to reach inhibitory concentrations at the target PBPs. Thus, the cell wall barrier alone cannot account for resistance to beta-lactam antibiotics.

The cell wall barrier may contribute to resistance in concert with β-lactamase (25) by limiting the entry of beta-lactam antibiotics. A small amount of enzyme may hydrolyze antibiotic, even a poor substrate, faster than it can enter the cell. If so, inhibition of the enzyme should restore antibacterial activity by permitting active drug to reach its target. *M. tuberculosis* produces a Bush and Sykes class 2b broad-spectrum β-lactamase that hydrolyzes penicillins and cephalosporins but not carbapenems (4, 5, 19, 31, 36), and it is inhibited by clavulanic acid, sulbactam, and the β-lactamase-stable antistaphylococcal penicillins (20, 21, 36). Confirming results previously reported by Zhang et al. (36) for strain H37Rv, we demonstrated that the β-lactamase activity of its avirulent counterpart, strain H37Ra, was inhibited by clavulanic acid and sulbactam, with IC_{50}s for hydrolysis of nitrocephin of 0.2 and 1.3 μg/ml, respectively. These concentrations are well within the therapeutic ranges of these inhibitors (1, 11).

In other bacterial species that are susceptible to beta-lactam antibiotics, the high-molecular-mass PBPs are the critical targets, and the binding of low-molecular-mass PBPs is not required for antibacterial activity (3, 13, 32). Assuming that this is also the case for *M. tuberculosis*, ampicillin, amoxicillin, cefoxitin, ceftriaxone, cephaloridine, and imipenem should be active, because their IC_{50}s were within the range of clinically achievable concentrations and within the range observed for PBPs of other susceptible bacterial species (3). MICs were determined by the broth dilution method (14, 28) to confirm the predicted activity against *M. tuberculosis* and to assess which PBPs were the likely targets mediating the antibacterial effect. Because MICs require at least 5 to 6 days to reach the endpoint and since beta-lactam antibiotics are relatively unstable compounds, loss of active drug during extended incubation was determined. Clavulanic acid, cefoperazone, cephalothin, and imipenem were the least stable compounds, with half-lives of less than 2 days (Table 3). Imipenem was particularly unstable, with a half-life of 0.4 days, yet it was the most active single agent, with MICs of ≤4 μg/ml for all strains (Table 3). This is consistent with the high affinity of all four PBPs for imipenem and its resistance to hydrolysis by penicillinases and cephalosporinases. Ceftriaxone and cephaloridine had variable activities (MICs, 16 μg/ml or less for two and three strains, respectively). Ampicillin and amoxicillin were ineffective as single agents because these drugs are not β-lactamase stable (6, 10, 18, 19, 29, 33), but in the presence of β-lactamase inhibitors, these antibiotics were quite active.

Which PBPs were likely to be critical targets mediating the antibacterial effects of the beta-lactam antibiotics could be

TABLE 2. Permeability coefficients of mycobacterial cell wall by beta-lactam antibiotics

Antibiotic	Hydrophobicity [$\log_{10} P_u$ (octanol)][a]	Permeability coefficient (nm/s)	
		M. chelonei PS4770[b]	*M. tuberculosis* H37Ra
Cephaloridine	2.0	1.0	9.4
Cephalothin	1.1	0.3	2.2
Cefazolin	−0.25	0.2	1.6
Cephacetrile	−0.45	0.2	2.4
Amoxicillin			2.3
Ampicillin			3.1
Benzylpenicillin			4.9
Carbenicillin			2.1

[a] Expressed as an octanol-water partition coefficient (P_u), as in references 17 and 26.
[b] See reference 17.

TABLE 3. Beta-lactam antibiotic MICs and half-lives for *M. tuberculosis* H37Ra, H37Rv, and five clinical isolates

Antibiotic	MIC (μg/ml)							Half-life (days)
	H37Ra	H37Rv	1	2	3	4	5	
Ampicillin	>16	>16	>16	16	>16	>16	>16	3.2
Amoxicillin	>16	>16	>16	16	>16	>16	>16	5.4
Nafcillin	>16	>16	>16	>16	>16	>16	>16	>6
Clavulanate	>16	16	>16	>16	>16	>16	>16	1.4
Sulbactam	>16	>16	>16	>16	>16	>16	>16	>6
Cefoperazone	>16	>16	>16	>16	>16	>16	>16	1.7
Cefoxitin	>16	>16	>16	>16	>16	>16	>16	3.5
Ceftriaxone	1	>16	>16	16	>16	>16	>16	>6
Cephaloridine	8	16	>16	8	>16	>16	>16	>6
Cephalothin	>16	>16	>16	>16	>16	>16	>16	1.2
Imipenem	4	4	4	4	4	4	2	0.4
Ampicillin + 10 μg of sulbactam per ml	≤0.1	≤2	≤2	≤2	≤2	≤2	ND[a]	
Amoxicillin + 8 μg of clavulanate per ml	0.5	≤2	≤2	≤2	≤2	≤2	≤2	
Cefoxitin + 8 μg of clavulanate per ml	8	8	8	ND	4	ND	8	
Ceftriaxone + 8 μg of clavulanate per ml	ND	4	4	≤2	4	≤2	4	
Nafcillin + 8 μg of clavulanate per ml	>32	>32	>32	ND	ND	ND	ND	

[a] ND, not done.

inferred by comparing MICs with PBP IC_{50}s. PBP 4 is not a critical target for beta-lactam antibiotics, because the antibacterial effect occurred at concentrations well below the IC_{50}s. PBPs 1, 2, and 3, like high-molecular-mass PBPs in other bacterial species, appear to be the critical targets. The lack of an antibacterial effect of clavulanic acid, sulbactam, or nafcillin, which are not hydrolyzed by *M. tuberculosis* β-lactamase (on the contrary, they are inhibitors of it), suggests that binding of PBP 2 or PBP 1 is necessary, but not sufficient, for antimycobacterial activity and that binding of PBP 3 is also required. The lack of an antibacterial effect with cefoperazone or cephalothin, which bound to PBP 3 but not to PBPs 1 and 2, indicates that binding of PBP 3 alone also is not sufficient.

The inactivity of cephaloridine was not unexpected because it is relatively rapidly hydrolyzed by *M. tuberculosis* β-lactamase (6, 10, 18, 19, 29, 33). Surprisingly, cefoxitin and ceftriaxone, both of which are relatively β-lactamase-stable compounds, were ineffective, despite favorable IC_{50}s of the high-molecular-mass PBPs. We hypothesized that the mycobacterial β-lactamase, like the β-lactamases produced by aerobic gram-negative bacilli, was hydrolyzing these drugs as they slowly entered the cell (25). Consistent with this hypothesis, the MICs of cefoxitin were reduced from >16 μg/ml to 4 to 8 μg/ml by the addition of clavulanic acid, and ceftriaxone MICs were reduced to ≤2 to 4 μg/ml (Table 3). This was not simply an additive effect unrelated to β-lactamase inhibition, because clavulanic acid did not reduce nafcillin MICs. The lack of activity of the clavulanate-nafcillin combination is additional evidence that PBP 3 is a critical target, because neither agent binds PBP 3 particularly well.

Since *M. tuberculosis* is able to parasitize macrophages, a desirable feature of an antituberculous agent is its ability to penetrate into these cells to eliminate intracellular organisms. The elimination of intracellular *M. tuberculosis*, presumably through penetration of drug into macrophages, was assessed by quantitative culture for viable mycobacteria that had been ingested by the mouse peritoneal macrophage cell line J774. Amoxicillin at 8 μg/ml plus either clavulanate or sulbactam at 4 μg/ml significantly reduced mycobacterial counts compared with control counts (Fig. 2). Sulbactam, clavulanic acid, or amoxicillin alone did not significantly reduce bacterial counts. Antibacterial activity was lost at concentrations of 2 μg of amoxicillin per ml plus 1 μg of inhibitor per ml.

It is intriguing that *M. tuberculosis* may be susceptible to the

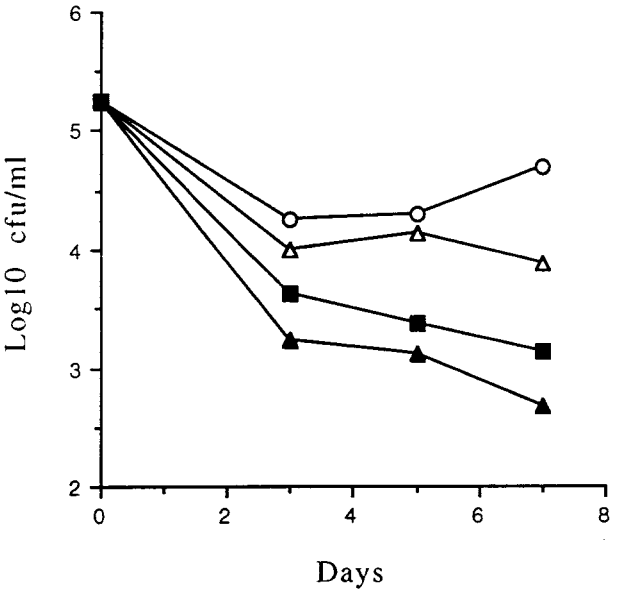

FIG. 2. Reduction of mycobacterial cell counts for J774 cells infected with *M. tuberculosis* H37Ra. Data are means of two separate experiments. ○, control (cells were incubated in drug free medium); △, 8 μg of amoxicillin per ml was added; ▲, amoxicillin plus 4 μg of clavulanic acid per ml; ■, amoxicillin plus 4 μg of sulbactam per ml. $P < 0.005$ for control versus amoxicillin plus clavulanic acid; $P < 0.005$ for control versus amoxicillin plus sulbactam; $P < 0.005$ for amoxicillin versus amoxicillin plus clavulanic acid; $P < 0.05$ for amoxicillin versus amoxicillin plus sulbactam; $P > 0.05$ for amoxicillin versus control (P values were determined by analysis of variance with Bonferoni correction). In separate experiments, neither clavulanic acid nor sulbactam alone had antibacterial activity (data not shown).

most extensively developed, highly effective, widely used, and nontoxic class of antimicrobials, agents as the present studies have indicated. The present studies are the first to examine each of the factors that together determine the susceptibilities of *M. tuberculosis* isolates to beta-lactam antibiotics. Binding to target PBPs occurred within the therapeutically achievable concentration ranges of several antibiotics. The penetration of beta-lactam antibiotics through the cell wall was similar for a variety of compounds and was adequate to achieve the required intracellular drug concentrations. Overcoming β-lactamase hydrolysis appeared to be particularly important for activity, and *M. tuberculosis* β-lactamase definitely should be studied further. Imipenem, amoxicillin-clavulanate, and ampicillin-sulbactam were the most active agents in PBP assays and in vitro. These drugs are excellent candidates for further evaluation in animal models and in patients for the treatment of tuberculosis.

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REFERENCES

1. **Adam, D., I. de Visser, and P. Koeppe.** 1982. Pharmacokinetics of amoxicillin and clavulanic and administered alone and in combination. Antimicrob. Agents Chemother. **22:**353–357.
2. **Barnes, P., and S. Barrows.** 1993. Tuberculosis in the 1990s. Ann. Intern. Med. **119:**400–410.
3. **Bryan, L., and A. Godfrey.** 1991. Beta-lactam antibiotics: mode of action and bacterial resistance, p. 599–664. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 3rd ed. The Williams & Wilkins Co., Baltimore.
4. **Bush, K.** 1989. Characterization of beta-lactamases. Antimicrob. Agents Chemother. **33:**259–263.
5. **Bush, K.** 1989. Characterization of beta-lactamases: groups 1, 2a, 2b, and 2b′. Antimicrob. Agents Chemother. **33:**264–270.
6. **Casal, M., F. Rodriguez, M. Benavente, and M. Luna.** 1986. In vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium fortuitum*, and *Mycobacterium chelonei* to Augmentin. Eur. J. Clin. Microbiol. **5:**453–454.
7. **Chambers, H., B. Hartman, and A. Tomasz.** 1985. Increased amounts of a novel penicillin-binding protein in a strain of methicillin-resistant *Staphylococcus aureus* exposed to nafcillin. J. Clin. Invest. **76:**325–331.
8. **Cynamon, M. H., and G. S. Palmer.** 1983. In vitro activity of amoxicillin in combination with clavulanic acid against *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **24:**429–431.
9. **Fattorini, L., G. Orefici, S. H. Jin, G. Scardaci, G. Amicosante, N. Franceschini, and I. Chopra.** 1992. Resistance to beta-lactams in *Mycobacterium fortuitum*. Antimicrob. Agents Chemother. **36:**1068–1072.
10. **Finch, R.** 1986. Beta-lactam antibiotics and mycobacteria. J. Antimicrob. Chemother. **18:**6–8.
11. **Foulds, G., J. Stankewich, D. Marshall, M. O'Brien, S. Hayes, D. Weidler, and F. McMahon.** 1983. Pharmacokinetics of sulbactam in humans. Antimicrob. Agents Chemother. **23:**692–699.
12. **Freiden, T., T. Sterling, A. Pablos-Mendez, J. Kilburn, G. Cauthen, and S. Dooley.** 1993. The emergence of drug-resistant tuberculosis in New York City. N. Engl. J. Med. **328:**521–526.
13. **Ghuysen, J.-M.** 1991. Serine beta-lactamases and penicillin-binding proteins. Annu. Rev. Microbiol. **45:**37–67.
14. **Inderlied, C.** 1991. Antimycobacterial agents: in vitro susceptibility testing, spectrums of activity, mechanisms of action and resistance, and assays for activity in biological fluids, p. 134–197. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 3rd ed. The Williams & Wilkins Co., Baltimore.
15. **Iseman, M.** 1994. Evolution of drug-resistant tuberculosis: a tale of two species. Proc. Natl. Acad. Sci. USA **91:**2428–2429.
16. **Jarlier, V., L. Gutmann, and H. Nikaido.** 1991. Interplay of cell wall barrier and β-lactamase activity determines high resistance to β-lactam antibiotics in *Mycobacterium chelonae*. Antimicrob. Agents Chemother. **35:**1937–1939.
17. **Jarlier, V., and H. Nikaido.** 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J. Bacteriol. **172:**1418–1423.
18. **Kasik, J.** 1965. The nature of mycobacterial penicillinase. Am. Rev. Respir. Dis. **91:**117–119.
19. **Kasik, J.** 1979. Mycobacterial beta-lactamases, p. 339–350. *In* J. Hamilton-Miller and J. Smith (ed.), Beta-lactamases. Academic Press, London.
20. **Kasik, J., M. Weber, and P. Freehill.** 1967. The effect of the penicillinase-resistant penicillins and other chemotherapeutic substances on the penicillinase of the R_1R_v strain of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **95:**12–19.
21. **Kasik, J., M. Weber, E. Winberg, and W. Barclay.** 1966. The synergistic effect of dicloxacillin and penicillin G on murine tuberculosis. Am. Rev. Respir. Dis. **94:**260–261.
22. **Laemmli, U.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Laskey, R., and A. Mills.** 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56:**335–341.
24. **Mizuguchi, Y., M. Ogawa, and T. Udou.** 1985. Morphological changes induced by β-lactam antibiotics in *Mycobacterium avium-intracellulare* complex. Antimicrob. Agents Chemother. **27:**541–547.
25. **Nikaido, H.** 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33:**1831–1836.
26. **Nikaido, H., E. Rosenberg, and J. Foulds.** 1983. Porin channels in *Escherichia coli*: studies with beta-lactams in intact cells. J. Bacteriol. **153:**232–240.
27. **Rosenblatt, J.** 1991. Antimicrobial susceptibility testing of anaerobic bacteria, p. 120–133. *In* V. Lorian (ed.), Antibiotics in Laboratory Medicine, 3rd ed. The Williams & Wilkins Co., Baltimore.
28. **Siddiqui, S.** 1989. BACTEC TB system products and procedure manual, section IV. Drug susceptibility testing. Becton Dickinson, Towson, Md.
29. **Sorg, T. B., and M. Cynamon.** 1987. Comparison of four beta-lactamase inhibitors in combination with ampicillin against *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **19:**59–64.
30. **Trias, J., V. Jarlier, and R. Benz.** 1992. Porins in the cell wall of mycobacteria. Science **258:**1479–1481.
31. **Watt, B., J. Edwards, A. Rayner, A. Grindey, and G. Harris.** 1992. In vitro activity of meropenem and imipenem against mycobacteria: development of a daily antibiotic dosing schedule. Tubercule Lung Dis. **73:**134–136.
32. **Waxman, D., and J. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. Annu. Rev. Biochem. **52:**825–869.
33. **Wong, C., G. Palmer, and M. Cynamon.** 1988. In vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium kansasii* to amoxycillin and ticarcillin in combination with clavulanic acid. J. Antimicrob. Chemother. **22:**863–866.
34. **Wyke, A.** 1984. Isolation of five penicillin-binding proteins from *Staphylococcus aureus*. FEMS Microbiol. Lett. **22:**133–138.
35. **Yajko, D., P. Nassos, C. Sanders, and W. Hadley.** 1989. Killing of antimycobacterial agents of AIDS derived strains of *Mycobacterium avium* complex inside cells of the mouse macrophage cell line J774. Am. Rev. Respir. Dis. **140:**1198–1203.
36. **Zhang, Y., V. Steingrube, and R. Wallace.** 1992. Beta-lactamase inhibitors and the inducibility of the beta-lactamase of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **145:**657–660.