

Successful Single-Dose Amoxicillin Prophylaxis Against Experimental Streptococcal Endocarditis: Evidence for Two Mechanisms of Protection

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Amoxicillin prophylaxis against experimental endocarditis due to one nontolerant and two tolerant strains of streptococci was studied in rats. Single-dose amoxicillin protected against the two tolerant strains in animals challenged with the 90% infective dose (ID_{90}), but protection diminished with increasing inoculum sizes. Protection against the nontolerant strain was successful with inocula that were 100- and 1,000-fold larger than the ID_{90} . Close correlation existed between the speed of bacterial killing in vitro, the time of exposure to bactericidal levels in vivo, and the range of inocula against which prophylaxis was effective. Amoxicillin seemed to protect by at least two mechanisms. (1) When in vitro tests indicated adequate bacterial killing, protection was independent of the inoculum size and was probably conferred by bacterial killing. (2) When in vitro tests indicated bacterial inhibition but not killing, protection was inoculum-dependent and was probably mediated by inhibition of bacterial adherence.

Preexisting endothelial cardiac lesions may be colonized during bacteremia, resulting in bacterial endocarditis. It has been recommended that patients with known cardiac lesions receive prophylactic antibiotics before undergoing procedures that might cause bacteremia [1, 2]. Because clinical trials of antibiotic prophylaxis in humans cannot be conducted for both ethical and statistical reasons [3], the question of which antibiotic and what dosage to use has been studied mainly in animals. The rabbit model developed by Garrison and Freedman [4] was used to test several prophylactic antibiotic regimens. These experiments suggested that bactericidal antibiotics would have to be given to obtain adequate prophylaxis and that a synergistic combination of antibiotics would be more effective than a single agent [5]. In rabbits, parenteral penicillin and vancomycin have been shown to be effective in preventing bacterial endocarditis. However, some strains of viridans streptococci are relatively resistant to the bactericidal

activity of penicillin [6]. Indeed, when we measured the sensitivity to amoxicillin, (a well-absorbed penicillin advocated for prophylaxis in humans [7]) and to vancomycin (the most powerful prophylactic agent in rabbits) of streptococci isolated from patients with endocarditis, we found that eight of 21 strains were tolerant to amoxicillin (MBC:MIC ratio, >32 [8]) and that five of 20 were tolerant to vancomycin.¹ Therefore, prophylaxis against endocarditis by means of bacterial killing would have failed against these strains. However, in recent experiments using a rat model, we showed that vancomycin prevented endocarditis in the absence of bacterial killing [9]. Because amoxicillin is easier to administer than vancomycin, we investigated amoxicillin prophylaxis against experimental endocarditis caused by strains of streptococci that are variously sensitive to the bactericidal activity of amoxicillin.

Materials and Methods

Microorganisms. Three bacterial strains were used to produce endocarditis. (1) *Streptococcus intermedius*, also called *Streptococcus sanguis* [9],

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was originally isolated from a patient with endocarditis. This strain has been used in experiments on endocarditis in both rabbits [10] and rats [9, 11]. (2) *S. sanguis* biotype II was provided by Dr. D. T. Durack (Duke University Medical Center, Durham, N.C.) who had used it in his investigations of prophylaxis against endocarditis in rabbits [5, 12]. (3) *Streptococcus mitior* was isolated from a patient in Lausanne with endocarditis. It was chosen because, of 23 strains of α -hemolytic streptococci isolated from patients with endocarditis, it was the most sensitive to the bactericidal action of amoxicillin.²

Determination of MICs, MBCs, and rates of bactericidal activity. The MICs of amoxicillin and vancomycin were determined with a standard broth dilution technique [13] with an inoculum of 10^5 – 10^6 organisms from either an overnight or a log-phase culture. The MBCs were determined by subculturing on penicillinase-containing blood agar plates 0.1 ml of an undiluted sample, as well as 0.1 ml each of 10-fold and 100-fold dilutions, from each dilution of antibiotic showing no turbidity after 24 hr and 48 hr of incubation. After incubation for 48 hr, the number of colonies on each plate was counted, and the MBC was determined as the lowest dilution of antibiotic that showed 99.9% killing. Using 10-fold and 100-fold dilutions of the antibiotic-containing broth avoids carry-over of antibiotic, a phenomenon that can give falsely low MBCs.

The rates of bactericidal activity of 25 μ g of amoxicillin/ml against the three strains of streptococci were determined in trypticase soy broth (Difco Laboratories, Detroit) using 10^6 organisms from an overnight culture as the inoculum. A concentration of 25 μ g of amoxicillin/ml was chosen because it is similar to serum levels achieved in rats 30 min after iv injection of 40 mg of the drug/kg of body weight and in humans 2 hr after an oral dose of 3 g [7]. At various times after the inoculation of bacteria into the antibiotic-containing broth, 10-fold (10^{-1}), 1,000-fold (10^{-3}), and 100,000-fold (10^{-5}) dilutions of a 0.1-ml sample were subcultured on penicillinase-containing blood agar plates and incubated overnight before colony counts were determined.

Serum levels of antibiotics and serum bacte-

ricidal activity. Serum levels of antibiotics were determined by the standard agar diffusion technique, using *Bacillus subtilis* as the test organism and normal rat serum as the diluent [14]. The samples were taken 30 min and 1, 2, and 4 hr after administration of various iv doses of amoxicillin to groups of five rats. The serum bactericidal activity against each strain 30 min and 1, 2, and 4 hr after iv administration of amoxicillin to nine rats was determined by standard methods [8], using 10^5 organisms from an overnight culture as the inoculum. The serum bactericidal activity was defined as the highest serum dilution providing 99.9% killing (<100 surviving bacteria) after 18 hr of incubation.

Production of endocarditis and evaluation of infection. Sterile vegetations were produced in female Wistar rats (weight, 180–200 g) by a slight modification of a previously described method [11]. In brief, a polyethylene catheter (model no. PP 10; Portex, Hythe, Kent, England) was inserted through the aortic valve into the left ventricle via the right carotid artery and secured with a silk ligature. Twenty-four hours after catheterization, rats were injected in the tail vein with 0.5 ml of 0.85% NaCl containing inocula of various sizes (expressed in cfu) from overnight cultures of the test microorganisms. Rats were killed 72 hr thereafter, and 1 ml of blood was drawn from the inferior vena cava and plated on blood agar. Aortic vegetations were excised, weighed, homogenized in 1 ml of 0.85% NaCl, serially diluted, and plated. Colony counts were determined after 48 hr of incubation at 37 C. This method permitted the detection of 10^2 cfu/g of vegetation.

Prophylaxis of endocarditis with amoxicillin. Thirty minutes before bacterial challenge with various inocula of the three test microorganisms, groups of rats were injected iv with either 40 mg of amoxicillin/kg of body weight or 0.85% NaCl alone.

Influence of amoxicillin on in vitro adhesion of S. intermedius and S. sanguis to platelet-fibrin matrices. Experiments were performed using an in vitro assay system that simulates nonbacterial thrombotic endocarditis as described by Scheld et al. [15]. In brief, cultures of *S. intermedius* or *S. sanguis*, grown overnight in brain-heart infusion broth supplemented with 5% sucrose, were suspended in either 25 μ g of amoxicillin/ml

² See footnote 1.

diluted in phosphate-buffered saline (PBS) or in PBS alone at a concentration of 10^3 cfu/ml. These suspensions were immediately poured into Petri dishes containing platelet-fibrin matrices, incubated for 2 min at 37 C in a shaking incubator, and washed two times with amoxicillin or PBS for 5 min and a third time with PBS supplemented with 2,000 units of penicillinase (Difco)/ml for 5 min. The time of exposure to amoxicillin was chosen to simulate the mean duration of bacteremia after iv challenge [9], during which period prevention of adhesion has to occur if it is to prevent endocarditis. Penicillinase-containing blood agar was then poured over the matrices, and the adherent colonies were counted after 24 hr of incubation. The adherence ratio for each sample was defined as the number of cfu adherent to the matrix divided by the initial number of cfu in the inoculum.

Statistical evaluation. The χ^2 test with Yates's correction and Student's unpaired *t*-test were used for statistical comparisons.

Results

The MICs and MBCs and the MBC:MIC ratio of amoxicillin for the three test organisms are shown in table 1. Although the MBCs of *S. intermedius* and *S. sanguis* were lower when subcultured after 48 hr, both bacteria remained tolerant.

Serum levels of amoxicillin in rats. After iv administration of various doses of amoxicillin into rats, it was found that 40 mg/kg iv resulted in a mean \pm SD peak serum level of 23.7 ± 3 μ g/ml at 30 min. This level was similar to those achieved in humans following an oral dose of 3 g of amoxicillin [7]. The values 1, 2, and 4 hr after iv injection in the rat were 8.8 ± 1.0 μ g/ml, 3.5 ± 0.4 μ g/ml, and 0.6 ± 0.3 μ g/ml, respectively.

Serum bactericidal activity. In order to measure the maximum bactericidal activity to which streptococci were exposed, serum bactericidal activities were measured at the time of bacterial injection, that is, 30 min after iv injection of 40 mg of amoxicillin/kg. No serum bactericidal activity could be detected against *S. intermedius*. The serum bactericidal activity against *S. sanguis* was 1:2, and that against *S. mitior* was >1:256 (table 1). After 1 hr, no serum bactericidal activity was detected against either *S. intermedius* or *S. sanguis*, while that against *S. mitior* was 1:128.

Serum bactericidal activity of 1:8 against the latter strain persisted 4 hr after amoxicillin was administered iv.

Rate of in vitro killing of the three test strains. Figure 1 shows the rate of in vitro killing of the three strains by 25 μ g of amoxicillin/ml, a concentration similar to the serum level achieved in rats 30 min after iv administration of 40 mg/kg and in humans 2 hr after an oral dose of 3 g. One million *S. mitior* organisms/ml were killed in 2 hr. In contrast, no significant killing of *S. intermedius* and *S. sanguis* occurred during the first 6 hr of exposure. The rates of killing for the three strains were similar regardless of whether an overnight or a log-phase culture was used.

Natural history of experimental endocarditis and prophylaxis with amoxicillin. *S. intermedius* endocarditis. As shown in figure 2, top, the incidence of infection in control rats increased directly with inoculum size. The 90% infective dose (ID_{90}) was found to be 10^5 cfu. In rats that received amoxicillin prophylaxis and were challenged with this inoculum, protection was almost complete. However, in rats receiving larger inocula, the protective effect of amoxicillin was overcome.

In the animals with endocarditis despite prophylaxis with amoxicillin, the mean bacterial densities in the vegetations were similar to those found in control animals, suggesting that amoxicillin did not diminish the severity of endocarditis due to *S. intermedius* when it failed to prevent the endocardial infection.

S. sanguis endocarditis. As shown in figure 2, middle, *S. sanguis* was less effective in causing endocarditis in rats than *S. intermedius* (ID_{90} , 10^6 cfu). Only in rats challenged with 1 ID_{90} was amoxicillin fully effective in preventing endocarditis; efficacy decreased with increasing inoculum size. In those amoxicillin-treated rats in which endocarditis was produced by giving an inoculum 10 times higher than the ID_{90} , infection as judged by the mean bacterial densities in the vegetations was less severe than in control animals that received no antibiotic. Thus, unlike the case with *S. intermedius*, amoxicillin influenced not only the incidence but also the course of endocarditis caused by *S. sanguis*.

S. mitior endocarditis. In the case of *S. mitior*, the ID_{90} was 10^5 cfu (figure 2, bottom). In contrast to both *S. intermedius* and *S. sanguis*, *S. mitior* failed to produce a high incidence of endocarditis

Table 1. Relationship of the kinetics of killing by amoxicillin in vitro, exposure to bactericidal activity in vivo, and protection against endocarditis for three streptococcal strains in rats.

Organism	MIC	MBC		MBC:MIC ratio		SBA	Time (hr) for peak killing in vitro*	Time (hr) of exposure to MBC in vivo†	Inoculum-dependent prophylaxis	Postulated mechanism of protection
		24 hr	48 hr	24 hr	48 hr					
<i>Streptococcus intermedius</i>	0.125	≥128	8	1,024	64	<1:2	48	≤0.5	Yes	Decreased adherence
<i>Streptococcus sanguis</i>	0.008	16	1	2,000	125	1:2	24	<4	Yes	Decreased adherence
<i>Streptococcus mitis</i>	0.004	0.032	0.032	8	8	>1:256	2	>4	No	Bacterial killing

NOTE. MICs and MBCs are expressed as $\mu\text{g/ml}$. Serum bactericidal activity (SBA) was measured 30 min after iv administration of 40 mg of amoxicillin/kg of body weight (that is, at the time streptococci were injected iv to induce endocarditis).

* Time required for amoxicillin at concentrations simulating peak serum levels in rats to kill the streptococcal strain in vitro.

† Amount of time streptococcal strains were exposed to the MBC of amoxicillin (determined at 48 hr).

in amoxicillin-treated rats, even with inocula 100 or 1,000 times the ID_{90} . Furthermore, in rats that developed endocarditis despite prophylaxis, infection as judged by the mean bacterial densities in the vegetations was much less severe than that found in animals that had developed *S. sanguis* endocarditis despite prophylaxis (figure 2, middle).

Effect of amoxicillin on the in vitro adhesion of S. intermedius and S. sanguis to platelet-fibrin matrices. When incubated in vitro with 25 μg of amoxicillin/ml for a period of time simulating the mean duration of bacteremia in vivo [9], the adherence ratio (mean \pm SD of 12 determinations) was reduced from 0.0174 ± 0.0055 for control determinations (PBS alone) to 0.0038 ± 0.0009 for determinations with amoxicillin-incubated *S. intermedius*, a reduction of 78% ($P < 10^{-5}$). For *S. sanguis*, the mean adherence ratio fell from 0.0165 ± 0.002 in control determinations to 0.009 ± 0.001 for those with amoxicillin-incubated bacteria, a reduction of 44% ($P < 10^{-5}$) (data not shown).

Discussion

Previous studies in rabbits have stressed the need for a bactericidal antibiotic or combination of antibiotics to achieve adequate prophylaxis against endocarditis due to *S. sanguis*. It has been observed, however, that vancomycin, the most potent antibiotic in rabbits, may afford protection in the absence of bacterial killing both in rabbits and rats [9, 16]. The present study clarifies the conditions in which killing or alternative mechanisms may be operative in preventing endocarditis.

It is improbable that successful prophylaxis against endocarditis due to *S. intermedius* or *S. sanguis* with a short course of amoxicillin could have acted through bacterial killing. It took 24 hr or more for sustained concentrations of amoxicillin that simulate peak serum levels to kill both strains in vitro. In vivo, these peak concentrations were transient due to the rapid elimination of amoxicillin from the rat serum, thus leaving little time for killing of the infectious inoculum (table 1). In addition, only insignificant serum bactericidal activity was detected at the time of bacterial challenge following administration of amoxicillin. Thus, these results extend studies of vancomycin prophylaxis to additional streptococcal strains and

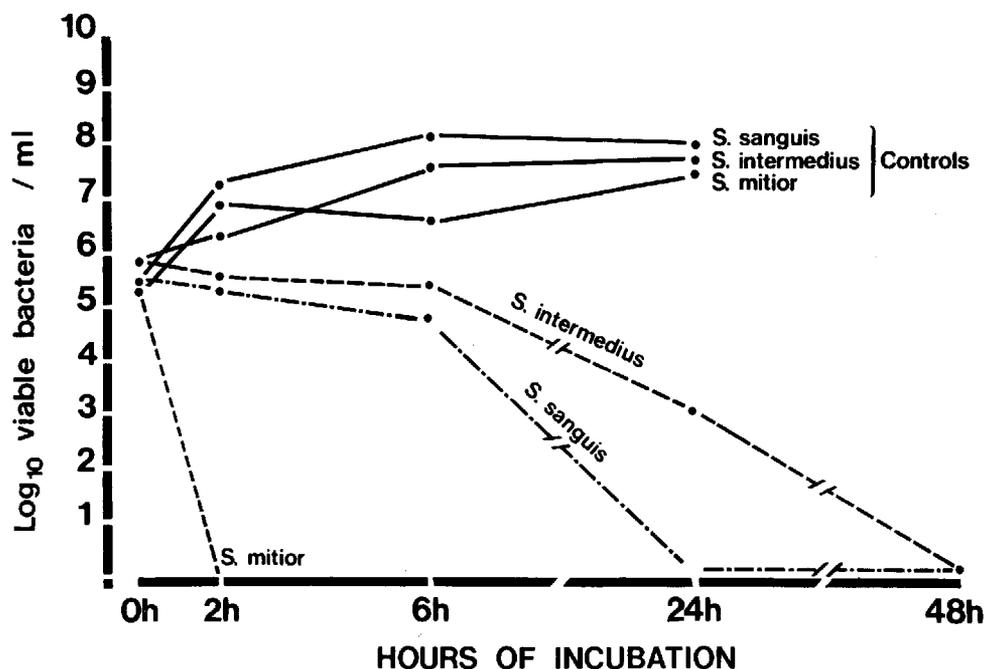


Figure 1. Rate of in vitro killing of *Streptococcus intermedius*, *Streptococcus sanguis*, and *Streptococcus mitior* by 25 µg of amoxicillin/ml (a concentration similar to serum levels in rats 30 min after a single dose [40 mg/kg of body weight] given iv).

to amoxicillin, which is more practical to administer than vancomycin. In these studies amoxicillin prevented endocarditis in the absence of killing, probably by inhibiting the adherence of *S. sanguis* to the sterile vegetation [9, 16]. Several reports suggest that nonbactericidal concentrations of β -lactam antibiotics, which, like vancomycin, act on cell-wall synthesis, inhibit the adherence of bacteria in vitro to platelet-fibrin clots [16–19]. In the present experiments, amoxicillin at concentrations simulating peak serum levels rapidly and efficiently inhibited the in vitro adherence of both *S. intermedius* and *S. sanguis* on platelet-fibrin matrices. Therefore, we postulate that a single dose of amoxicillin may have acted through the same mechanism as vancomycin in preventing endocarditis following challenge with bacteria that are only minimally sensitive to its bactericidal action.

In contrast to its effects on *S. intermedius* and *S. sanguis*, we found that the prophylactic activity of amoxicillin against *S. mitior* was demonstrable with inoculum sizes that were 1,000 times the ID_{90} . This difference in ability of bacteria to produce endocarditis after exposure to prophylaxis with amoxicillin may be related to the different kinetics

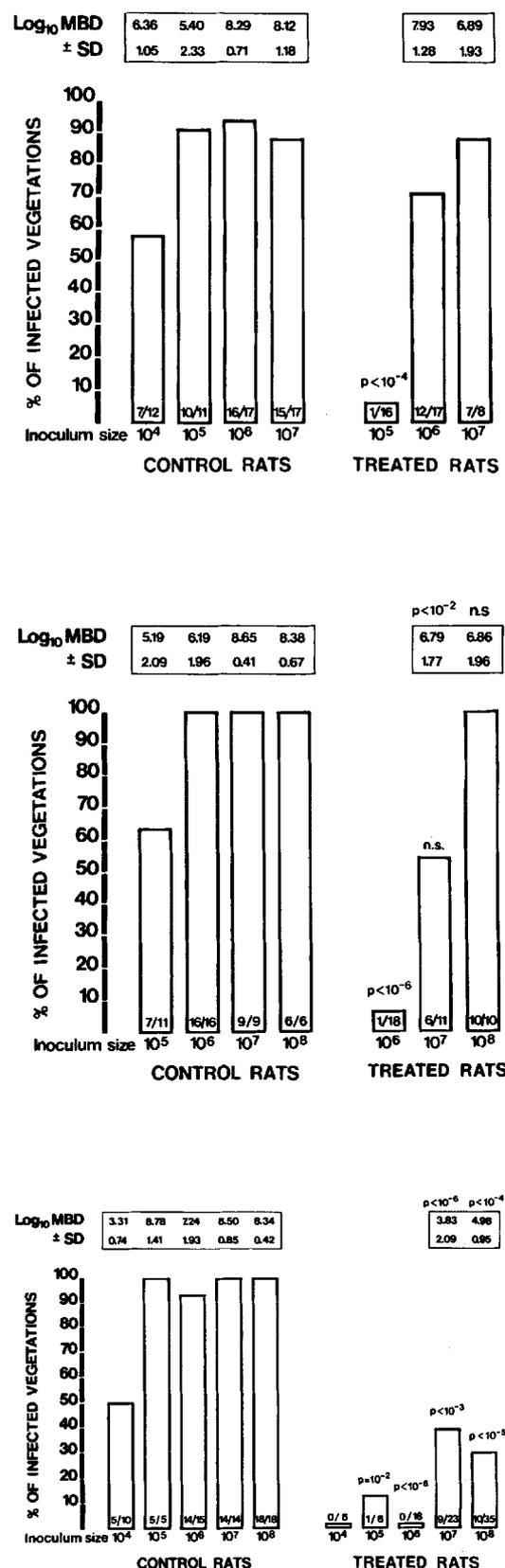
of killing among the strains. Only *S. mitior* was exposed to a high serum bactericidal activity ($>1:256$) after iv administration of amoxicillin. Furthermore, *S. mitior* was the only streptococcal species of the three tested that was exposed in vivo to concentrations of amoxicillin greater than its MBC for a longer period than was required to kill it in vitro (table 1). We interpret these findings as indicating that amoxicillin could have acted by two mechanisms when preventing endocarditis. (1) With *S. mitior*, an organism that was rapidly killed, amoxicillin may have protected primarily by killing. The protection afforded by killing was inoculum-independent, that is, not limited to the ID_{90} , but extending to inoculum sizes that were 1,000-fold greater. (2) With those strains that were only slowly killed by amoxicillin in vitro, protection may have been due to the prevention of bacterial adherence to vegetations rather than to actual bactericidal activity [9, 15–19]. Protection afforded by this mechanism was inoculum-dependent, that is, limited to the ID_{90} and decreasing with increasing inoculum sizes (table 1).

When inoculum sizes of *S. mitior* 10, 100, or 1,000 times larger than the ID_{90} were used to infect rats, the endocardial infection that developed in a

few rats three days after amoxicillin prophylaxis was less severe than in control rats. A similar phenomenon, although less pronounced, was observed following challenge with 10 times the ID_{90} of *S. sanguis*. Thus, bacteria that were not killed by amoxicillin appeared damaged sufficiently to adversely affect multiplication once endocardial infection was established, thus demonstrating a postantibiotic effect in vivo [20]. Further experiments have shown that this phenomenon is transient, since the few rats challenged with *S. mitior* that developed endocarditis despite prophylaxis had 10^9 cfu of *S. mitior*/g of vegetation when killed seven days after infection, a degree of infection similar to that found in control rats (data not shown).

The results of this study agree with those of our recent work showing that a short course of clindamycin prophylaxis successfully prevented endocarditis due to *S. sanguis*, *S. intermedius*, and *S. mitior* in rats when given in doses that produce serum levels equal to those achievable by oral administration in humans [21]. Since clindamycin is bacteriostatic, these experiments provide further evidence that killing is not essential for effective prophylaxis. The results of both studies appear to contradict those of previous observations in rabbits by Durack and Petersdorf [5] and Pelletier et al. [12], in which single injections of several antibiotics failed to prevent infection. However, the latter experiments were performed with an inoculum of 10^8 cfu of *S. sanguis*, an inoculum which was probably many times higher than the ID_{90} for rabbits. If so, our observations on the dependence of prophylaxis efficacy on inoculum size provides an explanation for this apparent

Figure 2. Incidence of endocarditis in control and amoxicillin-treated rats three days after bacterial challenge with (top) *Streptococcus intermedius*, (middle) *Streptococcus sanguis*, and (bottom) *Streptococcus mitior*. The mean (\pm SD) bacterial density (MBD) recovered from vegetations (expressed as cfu/g of vegetation) is indicated for each group. The no. of rats with infected vegetations/total no. of rats per group is indicated at the base of each column. To compare the incidence of endocarditis in control vs. treated rats, P values were calculated by χ^2 analysis with Yates's correction. To compare MBDs in control vs. treated rats challenged with the same inoculum size, P values were calculated by Student's unpaired t -test (n.s. = not significant).



contradiction. Rabbits challenged with many times the ID₉₀ would not be protected because the strain of *S. sanguis* used in these studies (provided to us by Dr. Durack) was not rapidly killed by the antibiotics tested for prophylaxis.

In considering the relevance of these results to prophylaxis against endocarditis in humans, we were interested to know which of the two prophylactic mechanisms we have observed was likely to be clinically operative. We found that one-third of streptococcal strains causing endocarditis were tolerant to amoxicillin and two-thirds were tolerant to vancomycin.³ This result suggests that, to prevent bacterial endocarditis due to these tolerant strains, these antibiotics would have to act by mechanisms other than killing. The present experiments in rats indicate that these alternative mechanisms protect against challenging inocula only as high as the ID₉₀. However, there are two reasons which suggest that the administration of 3 g of amoxicillin could prevent endocarditis in humans. (1) The magnitude of the bacteremia necessary to reach the ID₉₀ in rats is probably far greater than that present in humans after dental manipulation [22]. (2) In the present experiments, the plastic catheter was left in place before prophylaxis was tested; however, few patients at risk of developing endocarditis harbor an endovascular foreign body, and we have previously shown that removal of the catheter increases the effectiveness of prophylaxis with vancomycin 1,000-fold [11]. The results in our experimental model therefore suggest that, even in the absence of bacterial killing, endocarditis may be prevented by other mechanisms—possibly inhibition of adherence—and that these mechanisms may give the prophylactic regimen a margin of safety even after single-dose prophylaxis.

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³ See footnote 1.

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