Successful Prophylaxis of Experimental Streptococcal Endocarditis with Single-Dose Amoxicillin Administered after Bacterial Challenge

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Rats with catheter-induced aortic vegetations were challenged intravenously with various inoculum sizes of tolerant *Streptococcus sanguis* or *Streptococcus faecalis*. Single-dose amoxicillin (40 mg/kg) was given intravenously either 30 min before or 30–240 min after bacterial challenge. Prophylaxis of endocarditis against both strains was successful when the inocula used for challenge were in the range of the minimum inoculum producing bacterial endocarditis in 90% of control animals (ID_{90}) but was less effective or failed with larger inocula or when amoxicillin administration was delayed up to 240 min after bacterial challenge with *S. sanguis*. In a group of rats profoundly depleted of neutrophils by a rabbit anti-rat neutrophil serum given 30 min after challenge with *S. faecalis* at ID_{90}, single-dose amoxicillin administered simultaneously with the antiserum was protective, indicating that neutrophils were not required for successful endocarditis prophylaxis.

Prevention of bacterial endocarditis by prophylactic antibiotics is recommended in patients at risk who are undergoing dental or medical procedures that may produce bacteremia [1]. The recommendation advocates antibiotic administration before the procedure, followed by one or more subsequent doses. The rationale was to have bactericidal antibiotic levels at the time bacteria would reach the circulation [2]. However, several experimental studies have shown that prophylaxis could be achieved in the absence of bacterial killing [3, 4] possibly by inhibiting the adherence of bacteria to the damaged cardiac valves, reinforcing the recommendations of antibiotic administration before the procedure [5–7].

However, recent observations have challenged the role of inhibition of bacterial adherence as a likely mechanism of successful prophylaxis in the absence of bacterial killing [8]. Indeed, these observations suggest that prophylactic antibiotics operate by inhibiting the growth of bacteria that adhere to vegetations during the bacteremic phase, allowing as-yet-undefined host defense mechanisms to progressively clear these bacteria from the valves.

We reasoned that if inhibition of growth, not of adherence, was a likely mechanism by which antibiotics prevented endocarditis, antibiotics administered after the bacteremic phase might also be successful. Thus, we attempted to determine the efficacy of antibiotics administered after the bacteremic phase in preventing endocarditis and to investigate the role of neutrophils in the successful antibiotic prevention of endocarditis.

Materials and Methods

Microorganisms. Two strains of streptococci, one *Streptococcus sanguis* and one *Streptococcus faecalis*, isolated from patients with bacterial endocarditis were used.

The minimum inhibitory concentrations (MIC) of amoxicillin were determined by the broth macrodilution technique using an inoculum of 10^5 cfu/ml of each strain from an overnight culture. The minimum bactericidal concentrations (MBC) were determined by subculturing on blood-agar plates supplemented with penicillinase (Difco, Detroit), 100 μl from each dilution of antibiotic showing no turbidity after 18 h of incubation.

Killing curves were determined in Mueller-Hinton broth (GIBCO, Paisley, Scotland) with an inoculum of 10^6 cfu/ml of each strain from an overnight culture using concentrations of amoxicillin of 10 μg/ml (corresponding to 10 times the MIC) and 100 μg/ml (corresponding to a concentration above the maximum peak serum value obtained immediately after an intravenous [iv] injection of 40 mg/kg of amoxicillin).

Amoxicillin serum levels and serum inhibitory and bactericidal titers. Serum levels of amoxicillin were determined 2, 30, 60, 120, and 240 min after injection of 40 mg/kg iv amoxicillin in catheterized rats by a standard agar diffusion technique. *Bacillus subtilis* was used as the test organism and normal rat serum was used as the diluent [9].

The serum inhibitory and bactericidal titers against *S. sanguis* and *S. faecalis* 30 min and 2, 4, and 6 h after iv administration of 40 mg of amoxicillin/kg of body weight were determined in three rats by standard methods [9] with an inoculum of 10^6 *S. sanguis* and *S. faecalis*. Subcultures were performed on penicillinase-containing blood agar (Bactopenase, 5×10^6 IU/l; Difco). The serum inhibitory titer was the highest dilution of serum inhibiting visible bacterial growth, and the serum bactericidal titer was the highest dilution

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of serum providing 99.9% killing of the original inoculum after incubation for 18 h.

Animal model—production of endocarditis. Sterile aortic vegetations were produced in female Wistar rats (180–200 g) by a method previously described [4]. In brief, a polyethylene catheter was placed through the right carotid artery across the aortic valve and secured with a silk ligature.

Twenty-four hours after catheterization, rats were injected in the tail vein with 0.5 ml of various amounts of bacteria from an overnight culture (bacterial challenge). Inoculum sizes were 10^6, 10^7, 10^8 cfu in the experiments with *S. sanguis* and 10^4 or 10^5 cfu in the experiments with *S. faecalis*. The lowest inoculum of each strain corresponds to the minimum inoculum producing bacterial endocarditis in 90% of control animals (ID_{90}). In all experiments, animals were killed 3 days after bacterial challenge. Aortic vegetations were excised, homogenized in 1 ml of saline, serially diluted, and plated. Plates were counted 24–48 h after incubation at 37°C.

Effect of single-dose amoxicillin administered before or after bacterial challenge. For each inoculum of the two strains, groups of rats of the same experiment were given a single dose of iv amoxicillin (40 mg/kg) administered either 30 min before or at various times after bacterial challenge. Control animals were given iv saline. In all experiments, controls and treated rats were handled concurrently.

Production of a rabbit anti-rat neutrophil serum (ANS). Rat neutrophils were harvested from peritoneal exudate 4 h after intraperitoneal (ip) injection of 5 ml of a solution of 3% thioglycolate. The cells were briefly suspended in distilled water to hemolyze residual red cells, washed, and resuspended in Hanks' balanced salt solution. The final cell suspension consisted of >98% neutrophils [10, 11]. Ten New Zealand rabbits (2.5 kg) were injected iv with 7×10^7 rat neutrophils at days 1, 8, and 14. The serum was collected at day 28 and again 10 days after a fourth injection of rat neutrophils. The samples of serum were pooled, decomplemented by heating at 56°C for 30 min, absorbed against rats erythrocytes for 30 min (in order to avoid hemolysis when injected in rats), and stored in aliquots at −80°C. Control serum (CS) was collected from nonimmunized rabbits and processed in the same way.

ANS administration and neutrophil counts. ANS or CS was injected in catheterized rats 30 min after iv bacterial challenge with 10^4 cfu of *S. faecalis* and the neutrophil counts determined at 5 and 30 min and 3, 6, 24, 48, and 72 h thereafter. ANS or CS was injected simultaneously iv and ip in a volume of 1 ml at each site of administration. Blood cells counts were performed by an automatic analyzer (Sysmex CC-800; TOA Medical Electronics, Japan).

Effect of neutrophil depletion on the efficacy of amoxicillin administered after bacterial challenge. Twenty-four hours after catheterization, animals were challenged with 10^4 cfu of *S. faecalis* (ID_{90}), and amoxicillin or saline was administered 30 min later (i.e., at a time when circulating bacteria could no longer be detected as previously shown [5]).

Rats given amoxicillin were divided into three groups and received amoxicillin alone, amoxicillin plus ANS, or amoxicillin plus CS. ANS and CS were injected at the same time as the antibiotic and administered both iv (1 ml) and ip (1 ml) to obtain a rapid and prolonged neutropenia. Control rats received CS.

Effect of neutrophil depletion on bacteremia. To detect if release of bacteria in the circulation from extravascular foci was expedited by neutropenia in animals given ANS and amoxicillin, blood (0.5 ml) was collected by jugular puncture 90 min after bacterial challenge and was plated onto penicillinase-supplemented blood-agar medium. The plates were incubated and examined after 48 h.

Statistical evaluation. The χ^2 test with Yates's correction was used for statistical comparisons.

Results

Minimum inhibitory and bactericidal concentrations. The MIC and MBC of amoxicillin were 0.032 and 128 μg/ml, respectively, for *S. sanguis* and 1 and 128 μg/ml for *S. faecalis*. These two strains were defined as tolerant to amoxicillin, with an MBC-to-MIC ratio well above 32.

Time-kill curve studies. No significant killing occurred during the first 6 h of exposure to amoxicillin for the two strains, with two different dosages of amoxicillin (figure 1). There was no difference between 10 and 100 μg/ml.

Serum antibiotic levels in rats and serum inhibitory and bactericidal titers against S. sanguis and S. faecalis. Serum levels of amoxicillin (mean ± SD for three rats at each time interval) after iv injection of 40 mg/kg amoxicillin were 188 ± 12.5 μg/ml at 2 min, 40 ± 5.8 μg/ml at 30 min, 12.7 ± 2.9 μg/ml at 60 min, 1.9 ± 0.7 μg/ml at 120 min, and 0.6 ± 0.1 μg/ml at 240 min.

For *S. sanguis*, the mean serum inhibitory titer 30 min, 2 h, and 4 h after iv injection of amoxicillin was 1/32; after 6 h it was 1/8. For *S. faecalis*, the mean serum inhibitory titer 30 min after injection of amoxicillin was 1/4. No serum inhibitory activity was detectable at 2, 4, or 6 h. For both strains, no serum bactericidal activity could be detected at any time.

Effect of single-dose amoxicillin administered before or after bacterial challenge with S. sanguis. As previously observed [12], amoxicillin given 30 min before bacterial challenge was successful in preventing *S. sanguis* endocarditis, provided that the infectious dose was in the range of the ID_{90} (10^6–10^7

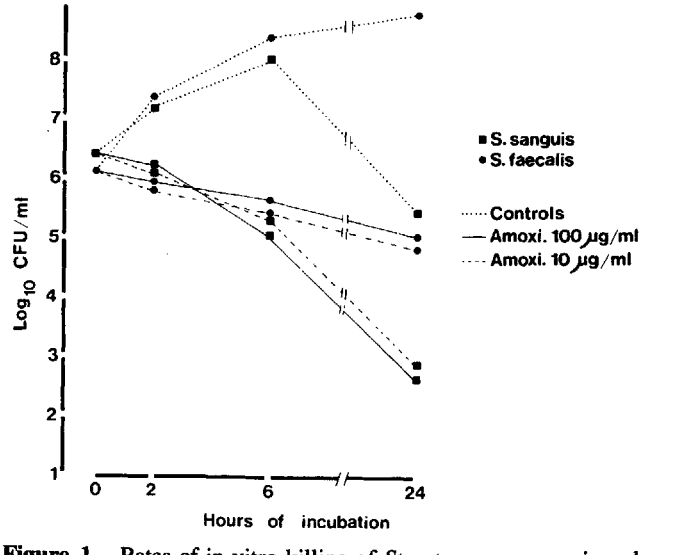

Figure 1. Rates of in vitro killing of *Streptococcus sanguis* and *Streptococcus faecalis* by 10 and 100 μg/ml of amoxicillin (Amoxi).

cfu) (figure 2). Amoxicillin failed with a higher inoculum size (10^8 cfu).

When amoxicillin was administered 30 min after bacterial challenge (i.e., when circulating bacteria were no longer detectable in the blood) or 120 min after challenge, excellent protection was also observed with inocula in the range of the ID_{90} but vanished with higher inocula. When amoxicillin was administered 240 min after challenge, no protection was observed.

Effect of single dose amoxicillin administered before and after bacterial challenge with S. faecalis. Results obtained were similar to those with *S. sanguis* (figure 3). Excellent protection was achieved with amoxicillin administered either 30 min before or 30–120 min after bacterial challenge with inoculum sizes not higher than the ID_{90}. With an inoculum 10 times the ID_{90}, the protective effect decreased substantially when amoxicillin was given either 30 min before or 120 min after bacterial challenge.

Effect of ANS on neutrophil counts of infected rats. Administration of ANS resulted in a rapid (<5 min) and profound (>99%) decrease in the number of circulating neutrophils lasting over 6 h (figure 4), which contrasted with an increased neutrophil count observed in rats given CS. The few residual neutrophils detected during the period of neutropenia showed marked morphologic alterations, suggesting possible functional impairment. Neutrophil counts returned to baseline values after 48–72 h. Lymphocyte and monocyte counts were not affected, but circulating platelet numbers were decreased about fivefold in parallel to neutrophil counts.

Blood cultures performed on penicillinase-supplemented agar plates 1 h after ANS injection in rats (i.e., 90 min after bacterial challenge) were sterile, providing evidence for the absence of secondary bacteremia during the neutropenic episodes.

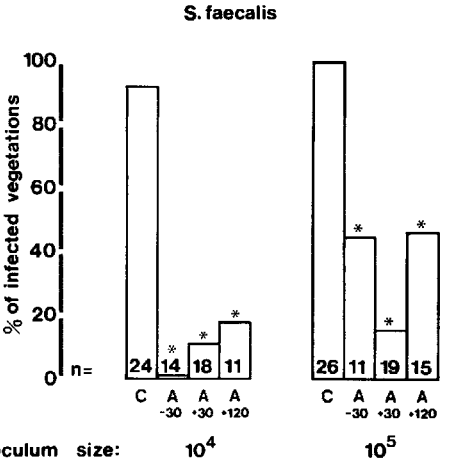

Figure 3. Incidence of endocarditis in control rats (C) and in rats given amoxicillin 30 min before (A−30) or 30–120 min after (A+30−A+120) bacterial challenge with various inocula of *S. faecalis*. See figure 2 legend for details.

Effect of single-dose amoxicillin administered 30 min after bacterial challenge with S. faecalis in neutropenic rats. Successful amoxicillin prophylaxis was achieved in rats with ANS-induced neutropenia as well as in rats with normal neutrophil counts. This indicated that neutrophils were not required for the successful protection conferred by amoxicillin given after *S. faecalis* challenge (figure 5).

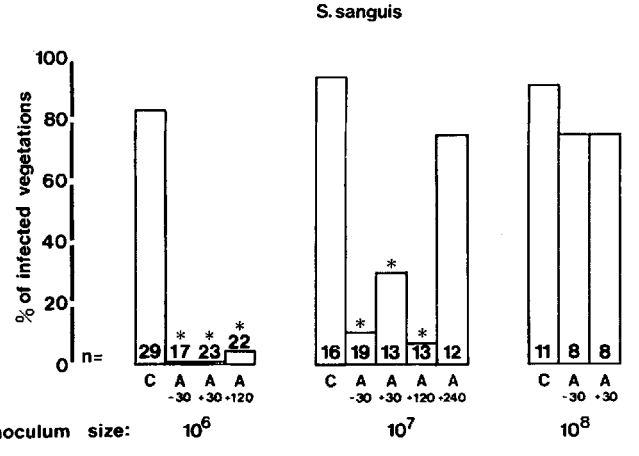

Figure 2. Incidence of endocarditis in control rats (C) and in rats given amoxicillin 30 min before (A−30) or 30–240 min after (A+30−A+240) bacterial challenge with various inocula of *S. sanguis*. *P* values were calculated by χ^2 analysis with Yates's correction; asterisk indicates $P < .05$ compared with controls. There were no significant statistical differences between A−30, A+30, and A+120.

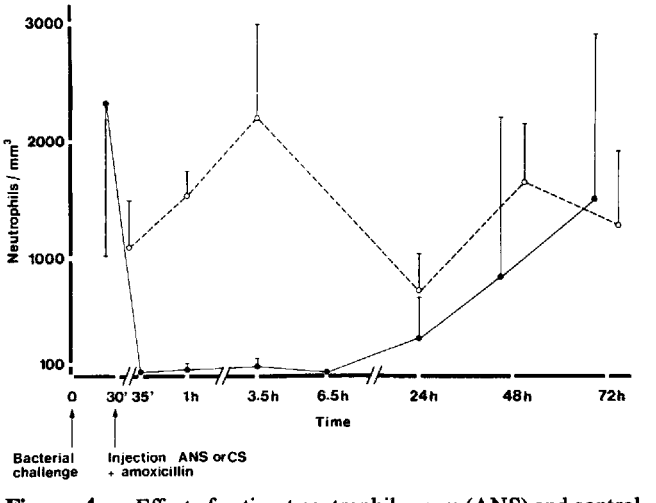

Figure 4. Effect of anti-rat neutrophil serum (ANS) and control serum (CS) on neutrophil counts of catheterized control (○) and ANS-treated (●) rats inoculated with 10^4 cfu of *S. faecalis*. Each point represents mean neutrophils ± SD (vertical lines) at time after bacterial challenge.

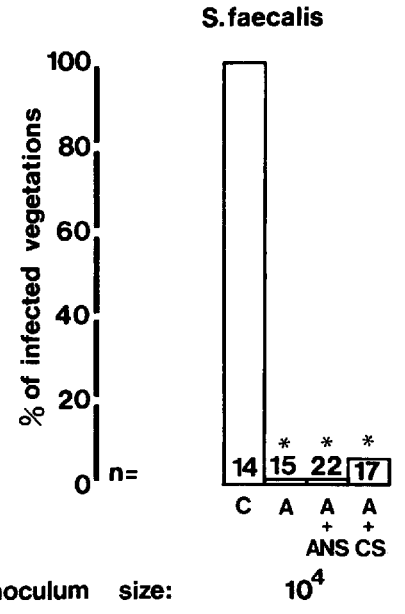

Figure 5. Incidence of endocarditis in rats challenged with 10^4 cfu of *S. faecalis* and given saline (C), amoxicillin alone (A), amoxicillin plus anti-rat neutrophil serum (A+ANS), or amoxicillin plus control serum (A+CS). Asterisk indicates $P < .05$ compared with controls.

Discussion

Several experimental studies have shown that single doses of amoxicillin or other antibiotics administered before bacterial challenge successfully prevented endocarditis in the absence of bacterial killing, provided that the inoculum size was in the range of the ID_{90} [3, 4, 8]. Under these circumstances, inhibition of bacterial adherence to the vegetations has been proposed as the main mechanism of action of antibiotics responsible for successful prevention of endocarditis.

Previous in vitro experiments indicated a decreased ability of bacteria treated with antibiotics to adhere to platelet-fibrin matrices mimicking endocardial vegetations, but experiments with animals injected with antibiotic-pretreated bacteria gave contradictory results [5–7]. Thus the biologic importance of inhibition of adherence by antibiotics was uncertain.

In a recent study, Moreillon et al. [8] showed that the protective effect of amoxicillin given before bacterial challenge was lost if amoxicillin was inactivated by penicillinase injected at the end of bacteremia, demonstrating that inhibition of adherence was not a likely mechanism of successful prophylaxis in these experiments. Moreover, these observations suggested that prolonged inhibition of bacterial growth on the surface of the vegetations was required for successful prevention of endocarditis.

In the present study, we observed that amoxicillin given 30 or 120 min after bacterial challenge was as effective as the antibiotic given before challenge in preventing *S. sanguis* and *S. faecalis* endocarditis. Thus, the observation that amoxicillin was effective if administered when the bacteria had already adhered to the vegetations and could no longer be recovered from the blood [5] strengthens the evidence that protection was not conferred by inhibition of adherence of the bacteria to the damaged valves. This confirms early experiments performed by Durack and Petersdorf [2], who reported protection in rabbits injected with streptococci and given high doses of procaine penicillin 30 min after bacterial challenge.

Detachment of the adherent bacteria from the vegetations through structural modification of the bacterial wall during exposure to circulating amoxicillin has been proposed as a possible mechanism for successful prophylaxis [7, 8]. In our experiments, however, blood cultures onto penicillinase-containing media performed after amoxicillin administration were sterile. Moreover, this hypothesis is not supported by our previous in vitro experiments in which bacteria attached to platelet-fibrin matrices did not show increased detachment when exposed to and washed with bacteriostatic concentrations of amoxicillin for 4 h, a period simulating the time of exposure to amoxicillin in vivo [8]. Therefore, detachment of adherent bacteria appears to be an unlikely mechanism by which bacteria are released from the vegetations. However, this cannot be totally ruled out, because of the methodologic difficulty of showing a phenomenon that implicates only a very small number of bacteria.

Bacterial killing has been considered as a potential mechanism of action of prophylactic antibiotic. However, this mode of action does not seem to account for the present results, because the streptococcal strains used were tolerant to amoxicillin, particularly *S. faecalis*, which is notoriously resistant to the bactericidal actions of β-lactams.

Moreover, no serum bactericidal activity was detected in vivo at any time. Killing curves performed in vitro with amoxicillin concentrations as high as 100 μg/ml showed no significant killing during the first 2 h of exposure and a reduction of <1 log after 6 h (figure 1). Therefore, these observations provide further evidence that mechanisms other than bacterial killing are involved in the successful antibiotic prophylaxis of endocarditis.

Previous experiments have shown that after the initial attachment of bacteria that follows the challenge, the bacterial counts at the surface of the vegetation decreased progressively during the first 2 h, even in the absence of prophylactic antibiotics [8]. From 2 h on, if residual organisms were still present, bacterial counts increased again because of resumed multiplication. In animals given amoxicillin 30 min before bacterial challenge and injected with increasing inoculum sizes up to the ID_{90}, this natural decrease in bacterial counts was prolonged beyond 2 h by the bacteriostatic action of amoxicillin, so that the bacteria were cleared from the vegetations and endocarditis could not develop later.

In the present experiments, successful prophylaxis was also observed in rats given amoxicillin up to 2 h after bacterial challenge. This effect however was limited to animals chal-

lenged with bacterial inocula not higher than the ID_{90} for both strains; also it vanished when amoxicillin administration was delayed up to 4 h after challenge with *S. sanguis* (figure 2). Prior experiments have shown that in control animals, bacterial growth on the vegetations has begun at 4 h and counts are 10-fold higher than counts at 30 min [8]. In fact, the counts at 4 h after injection of the ID_{90} were similar to those obtained at 30 min in animals injected with 10 times the ID_{90}. In the latter animals a higher rate of prophylaxis failure was observed compared with that in animals challenged with the ID_{90}. This might explain the present results in which single-dose amoxicillin administered 4 h after bacterial challenge with *S. sanguis* at the ID_{90} failed to prevent endocarditis. Successful prophylaxis could possibly be restored by using multiple doses of amoxicillin as demonstrated in animals challenged with inocula 10–1000 times the ID_{90} [13].

Since the clearance of bacteria from the vegetations appeared to be a likely mechanism operating in endocarditis prophylaxis, we further investigated whether the neutrophils would contribute to this effect and therefore be implicated in the success of amoxicillin in preventing endocarditis. We found that a profound and rapid neutropenia (figure 4) induced after the end of the bacteremia did not decrease the prophylactic efficacy of amoxicillin (figure 5). These experiments showed that the granulocytes were unlikely to play an important role in the clearance of bacteria from the vegetations, suggesting that either mere mechanical detachment of bacteria (but not detectable by blood culture) or possibly a platelet-mediated bactericidal activity [14] could be responsible for successful amoxicillin prophylaxis in the absence of bacterial killing.

In conclusion, the present study indicates that single-dose amoxicillin given after bacterial challenge can successfully prevent experimental endocarditis due to tolerant streptococcal strains, provided that the inoculum size is not greater than the ID_{90} and that antibiotic administration occurs within 2 h after bacterial challenge. Neutrophils do not appear to be a contributing factor to successful prophylaxis of experimental endocarditis.

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