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Single-Dose Rifampin Prophylaxis for Experimental Endocarditis Induced by High Bacterial Inocula of Viridans Streptococci

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In rats challenged with viridans streptococci poorly susceptible to antibiotic killing, single doses of antibiotics only prevent endocarditis induced by bacterial inoculum sizes that produce disease in 90% of control animals (ID_{90}): additional doses are required to protect against inocula exceeding the ID_{90}. We investigated whether single-dose rifampin would extend the efficacy of single-dose prophylaxis to inocula exceeding the ID_{90}. We used two strains of viridans streptococci highly susceptible to killing by rifampin and two resistant strains. All rats were injected with 10–1,000 times the ID_{90} of the four strains. Single-dose rifampin successfully prevented endocarditis due to all four strains. A few prophylaxis failures were observed after challenge with the two poorly susceptible strains, but in vivo emergence of resistant variants did not account for these failures. Thus, rifampin was the first antibiotic given as a single dose that successfully prevented experimental streptococcus endocarditis after challenge with high bacterial inocula.

Experimental streptococcus endocarditis can be successfully prevented by single doses of cell wall–active antibiotics like amoxicillin and vancomycin. When these antibiotics do not rapidly kill the bacteria used for challenge, however, the efficacy of single-dose prophylaxis is limited to the animals challenged with the lowest bacterial inoculum that infects 90% of controls (ID_{90}) [1–3]. Isolates of viridans streptococci poorly susceptible to the bactericidal action of cell wall–active antibiotics are increasingly reported from patients with endocarditis [4] and among other clinical isolates of α-hemolytic streptococci [5]. Against these bacteria, even a single dose of a synergistic combination of amoxicillin plus gentamicin failed to protect animals challenged with inocula exceeding the ID_{90}[6], and successful prevention of endocarditis could only be achieved by administering multiple doses of antibiotics that provide sustained blood levels after bacterial challenge (R. M., P. Francioli, M. P. G., unpublished observations).

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In the present experiments we tested the ability of rifampin to prevent experimental streptococcal endocarditis. We chose rifampin for two reasons. First, rifampin produces sustained blood levels in both rats and humans after single-dose administration [7, 8]. This pharmacological property permitted us to investigate whether single-dose rifampin, unlike single-dose amoxicillin or vancomycin, would prevent endocarditis induced by bacterial inocula higher than the ID_{90} of the test organisms. Second, rifampin has excellent in vitro activity against viridans streptococci [9]. We used single-dose rifampin prophylaxis against four different *Streptococcus* strains that were injected into rats at inocula 10–1,000 times the ID_{90} of the strains.

Materials and Methods

Microorganisms. Four strains of viridans streptococci isolated from patients with endocarditis were used. *Streptococcus intermedius* and *Streptococcus sanguis* (originally provided by Dr. D. Durack, Duke University Medical School, Durham, NC) have previously been used in experiments in rabbits [10, 11] and in rats [1–3, 6, 12]. In addition, two strains of *Streptococcus mitis*, *S. mitis* 1000 [6] and *S. mitis* 518, were selected. These strains were selected because, from 23 strains of α-hemolytic streptococci isolated from patients with bacterial endocarditis in our hospital, *S. mitis* 1000 and *S. mitis* 518 were the most susceptible to the bactericidal action of rifampin.

MICs, MBCs, and rates of killing. The MICs of rifampin (Ciba-Geigy, Basel, Switzerland) for the four test organisms were determined by a standard broth-dilution procedure [13] using an inoculum of 10^5 organisms from an overnight culture. The MBCs were determined by subculturing onto blood agar plates 10-fold and 100-fold broth dilutions of a 0.1-ml sample from each dilution showing no turbidity after 18 hr of incubation. This dilution procedure was used to avoid the carryover of antibiotic, a phenomenon that can give falsely low MBCs. After 48 hr of incubation 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 a concentration of 10 μg of rifampin/ml, we determined the rates of killing of the four test organisms in tryptic-soy broth (Difco, Detroit) by using a 10^5 inoculum from an overnight culture. A concentration of 10 μg of rifampin/ml was chosen because it was similar to peak serum levels in rats after receiving 20 mg of rifampin/kg sc and in humans after receiving 600 mg orally [8, 14]. At various times after inoculating the bacteria into the antibiotic-containing broth, 10^{-1}, 10^{-3}, and 10^{-5} dilutions of a 0.1-ml sample were subcultured onto blood agar plates and were incubated for 48 hr before colony counts were done.

Serum levels and activities. Serum levels of rifampin were determined by a standard agar-diffusion technique using *Sarcina lutea* (ATCC 9341) as the test organism and antibiotic medium no. 1 (Difco) [15]. Normal rat serum was used as the diluent. The samples were taken 2, 4, 8, 12, and 24 hr after an sc injection of 20 mg of rifampin/kg (water-soluble rifampin sodium salt; Ciba-Geigy) into groups of five rats for each time point.

Serum inhibitory activity (SIA) and serum bactericidal activity (SBA) for *S. intermedius*, *S. sanguis*, *S. mitis* 1000, and *S. mitis* 518 were determined 4, 8, 12, and 24 hr after an sc injection of 20 mg of rifampin/kg by standard methods [16], using an inoculum of 10^5 cfu/ml for each strain. Subcultures were performed on blood agar plates by plating 10-fold and 100-fold dilutions of a 0.1-ml sample of each dilution. The SIA was the highest serum dilution inhibiting visible bacterial growth, and the SBA was the highest serum dilution providing 99.9% killing of the original inoculum after 18 hr of incubation.

Rifampin-resistant variants in vitro. Because two strains of viridans streptococci, *S. sanguis* and *S. intermedius*, were not susceptible to the bactericidal action of rifampin, we investigated whether the lack of bacterial killing was due to the presence of rifampin-resistant subpopulations among these two strains. Experiments were performed as previously described to determine rates of killing. Briefly, using a 10^6 bacterial inoculum from an overnight culture, we determined the rates of killing of *S. intermedius* and *S. sanguis* by using a concentration of 10 μg of rifampin/ml. Before and after 12 and 24 hr of exposure to rifampin, 0.1 ml of undiluted 10^{-2}, 10^{-4}, and 10^{-6} dilutions of control and rifampin-exposed vials were subcultured onto blood agar plates and onto plates containing 1, 10, and 100 μg of rifampin/ml. The plates were incubated for 48 hr before colonies were counted. We used an additional 10^8 cfu inoculum to search for rifampin-resistant variants in the initial inoculum (i.e., without prior exposure to the drug).

Rifampin-resistant variants in vivo. After the rats were killed, 0.1-ml samples of undiluted homogenates of vegetation suspended in 0.85% NaCl from rats given rifampin prophylaxis were directly plated onto blood agar plates containing 1, 10, and 100 μg of rifampin/ml. The plates were incubated for 48 hr before the colonies were counted.

Adhesion of S. intermedius and S. sanguis to platelet-fibrin matrices. Experiments were performed by using an in vitro assay system that simulated nonbacterial thrombotic vegetations, as described previously [17]. The aim of these experiments was to investigate in vitro whether inhibition of bacterial adherence was a likely mechanism for successful rifampin prophylaxis against the two *Streptococcus* strains poorly susceptible to killing. In brief, cultures of *S. intermedius* and *S. sanguis* were grown overnight in tryptic-soy broth and suspended at a final concentration of 10^4 cfu/ml in PBS supplemented with 10 μg of rifampin/ml. Control bacteria were suspended in PBS alone. The suspensions were immediately poured into petri dishes containing the fibrin-platelet matrices and were incubated for 5 min at 37 C in a shaking incubator. The supernatant was then removed, and the matrices were washed two times for 5 min each with rifampin or PBS alone. This total time of exposure to rifampin (15 min) was chosen to simulate the mean duration of bacteremia after intravenous challenge, during which time inhibition of adherence has to occur if it is to prevent endocarditis [2]. The petri dishes were then washed 10 times for 1 min each with PBS alone,

a procedure used to rapidly and completely eliminate rifampin from the matrices.

Endocarditis. Sterile vegetations were produced in female Wistar rats (180–200 g) by a previously described method [1]. In brief, a polyethylene catheter was inserted via the right carotid artery through the aortic valve into the left ventricle and secured with a silk ligature. Twenty-four hours after this procedure, rats were injected iv with 0.5 ml of 0.85% NaCl containing 10^8 cfu of *S. mitis* 518, *S. intermedius*, or *S. sanguis* test organisms or containing 10^7 cfu of *S. mitis* 1000 from an overnight culture.

The ID_{90} was determined as previously described [3]. In brief, the ID_{90} of *S. intermedius* and *S. mitis* 1000 was 10^5 cfu/ml; of *S. sanguis*, 10^6 cfu/ml; and of *S. mitis* 518, 10^7 cfu/ml. Thus, the inocula used in the prophylaxis experiments were 1,000 times the ID_{90} of *S. intermedius*, 100 times the ID_{90} of *S. sanguis* and *S. mitis* 1000, and 10 times the ID_{90} of *S. mitis* 518.

For *S. mitis* 1000, *S. intermedius*, and *S. sanguis*, we have previously established [6] that single-dose amoxicillin prophylaxis failed against inocula higher than the ID_{90}. For *S. mitis* 518, we established a similar phenomenon by using an inoculum size of 10^8 cfu/ml.

Prophylaxis. Four hours before challenge with the four test organisms, groups of rats were injected sc with either 20 mg of rifampin/kg or 0.85% NaCl alone (controls). The 4-hr interval between rifampin injection and bacterial challenge was chosen so that bacteria were injected at the time of peak rifampin levels.

Evaluation of infection. Rats given 0.85% NaCl (controls) were killed 72 hr after bacterial challenge. Rats given rifampin prophylaxis were killed seven days after bacterial challenge, a period that permitted the full development of endocarditis if rifampin prophylaxis failed.

After the rats were killed, 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 us to detect 10^2 cfu of bacteria/g of vegetation.

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

Results

MICs, MBCs, and rates of killing. The MICs and MBCs, respectively, of rifampin for the four test organisms were 0.016 and 0.032 μg/ml for *S. mitis* 1000, 0.008 and 0.064 μg/ml for *S. mitis* 518, 0.032 and 16 μg/ml for *S. intermedius*, and 0.032 and 32 μg/ml for *S. sanguis*. Thus, the first two strains exhibited MBC values close to their MICs, in contrast to the latter two strains, which showed higher MBC values.

The rates of in vitro killing of the four test organisms by 10 μg of rifampin/ml (simulating peak serum levels 4 hr after prophylaxis) are shown in figure 1. Bacterial counts of both *S. intermedius* and *S. sanguis* were not significantly reduced by peak concentrations of rifampin over 24 hr of incubation,

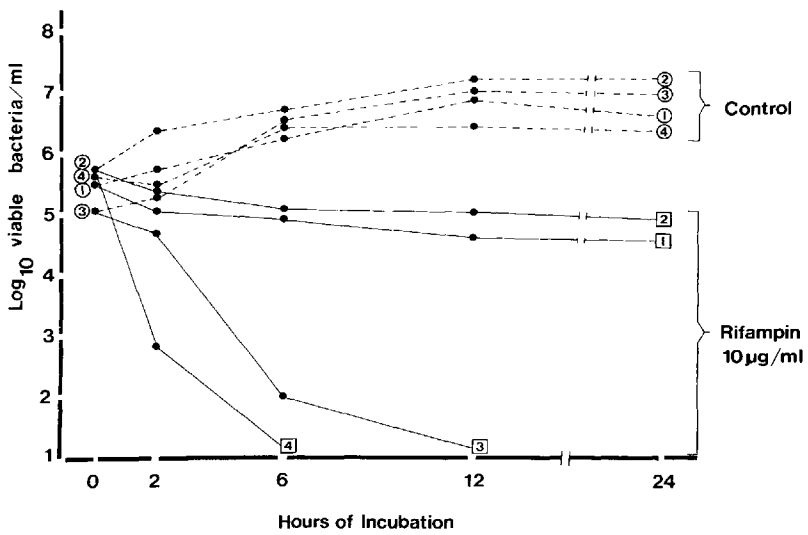

Figure 1. Rates of in vitro killing of four strains of viridans streptococci by 10 μg of rifampin/ml (a concentration similar to peak serum levels in rats 4 hr after receiving 20 mg of rifampin/kg sc). *1, S. sanguis*; *2, S. intermedius*; *3, S. mitis* 1000; and *4, S. mitis* 518.

whereas both strains of *S. mitis* were killed within 6 and 12 hr.

Rifampin-resistant variants of S. sanguis and S. intermedius. When *S. sanguis* was tested at an inoculum of 10^8 cfu, the frequency of resistant variants without prior exposure to rifampin was 10^{-7} (MIC, ≥256 μg/ml). At the 10^6 cfu inoculum, no rifampin-resistant variants could be detected without prior exposure to rifampin. In contrast, inocula exposed to rifampin for 12 hr showed a 10^{-3} frequency of resistant variants (MIC, ≥512 μg/ml). After 24 hr of exposure, all the colonies tested were highly resistant to rifampin.

When *S. intermedius* was tested at inocula of 10^6 and 10^8 cfu, no rifampin-resistant variants were detected without prior exposure to rifampin. When a 10^6 cfu inoculum was exposed to rifampin, however, the frequency of resistant variants was 10^{-2} (MIC, ≥1,024 μg/ml) after 12 hr of exposure, and all the colonies tested were highly resistant to rifampin (MIC, 512–1,024 μg/ml) after 24 hr of exposure.

Thus, the bacteriostatic effect of rifampin on *S. intermedius* and *S. sanguis*, observed when performing killing curves, was due to the selection of variants highly resistant to rifampin.

MICs and MBCs of 23 strains of viridans streptococci. The MICs of rifampin for all 23 strains of α-hemolytic streptococci isolated from patients with endocarditis were <0.064 μg/ml. Nineteen (83%) of 23 strains had MBC values that exceeded their MIC values >32 times.

Serum levels and activities. Two hours after rats were injected sc with 20 mg of rifampin/kg, the mean ± SD serum level was 9.1 ± 1.4 μg/ml; after 4 hr this value was 14.2 ± 1.26 μg/ml. Thus, peak serum levels were observed 4 hr after injection. The mean ± SD values 8, 12, and 24 hr after injection were 10.9 ± 1.22 μg/ml, 7.6 ± 0.8 μg/ml, and 0.4 ± 0.1 μg/ml, respectively.

After 36 rats were injected sc with 20 mg of rifampin/kg, no SBA against *S. intermedius* or *S. sanguis* could be detected at any time; however, SIAs of >1:16 could be detected until 12 hr after injection. Against *S. mitis* 518, the mean SBAs were 1:16, 1:8, and 1:4 at 4, 8, and 12 hr, respectively, after injecting five rats at each time point. Against *S. mitis* 1000, the SBAs were 1:8, 1:4, and 1:2 at 4, 8, and 12 hr after injection, respectively. Thus, only serum inhibitory titers were detected against the tolerant strains, whereas serum bactericidal titers were detected for up to 12 hr against the susceptible strains.

Prophylaxis with rifampin. Figure 2 shows the results of single-dose rifampin prophylaxis against the four *Streptococcus* strains. Rifampin completely prevented endocarditis induced by the two strains that were highly susceptible to killing by rifampin (*S. mitis* 1000 and *S. mitis* 518); the vegetations from all 38 animals were sterile seven days after bacterial challenge ($P < .001$ when compared with controls). Single-dose rifampin also significantly protected ($P < .001$ for both strains when compared with controls) against endocarditis induced by the two strains not killed by rifampin (*S. intermedius* and *S. sanguis*); however, prophylaxis failures were observed in seven of 25 rats challenged with *S. intermedius* and in four of 23 rats challenged with *S. sanguis*. The mean bacterial densities of the vegetations from the prophylaxis failures were significantly less than those in controls ($P < .003$), despite the fact that all animals given rifampin were killed seven days after administration of the antibiotic.

Adhesion of S. intermedius and S. sanguis to platelet-fibrin matrices. Because *S. intermedius* and *S. sanguis* were not killed by rifampin in vitro, we investigated whether successful prophylaxis with rifampin might have been mediated through diminished adherence of the organisms to cardiac valves. We therefore tested the influence of rifampin on the in vitro adherence of these two microorganisms to platelet-fibrin matrices. When incubated in vitro with 10 μg of rifampin/ml for 15 min, the adherence ratio (mean ± SD of 60 determinations) of *S. intermedius* was 0.091 ± 0.068 for controls (PBS alone) and 0.14 ± 0.088 for rifampin-incubated organisms ($P > .05$, Student's *t* test). For *S. sanguis*, the mean adherence ratio (60 determinations) was 0.072 ± 0.034 for controls and 0.063 ± 0.035 for rifampin-incubated organisms ($P > .05$, Student's *t* test). Thus, rifampin did not significantly influence the in vitro adhesion of *S. intermedius* or *S. sanguis* to platelet-fibrin matrices.

Rifampin-resistant variants in vivo. When samples of undiluted homogenates of the vegetations from the prophylaxis failures were plated onto rifampin-containing blood agar plates, none of the seven *S. intermedius* failures and only one of the four *S. sanguis* failures showed decreased susceptibility to rifampin. In the latter case, a few *S. sanguis* colonies grew on plates containing 1 μg of rifampin/ml, but none grew on plates containing 10 or 100 μg/ml. Thus, only one of 11 vegetations that was not protected from infection by single-dose rifampin pro-

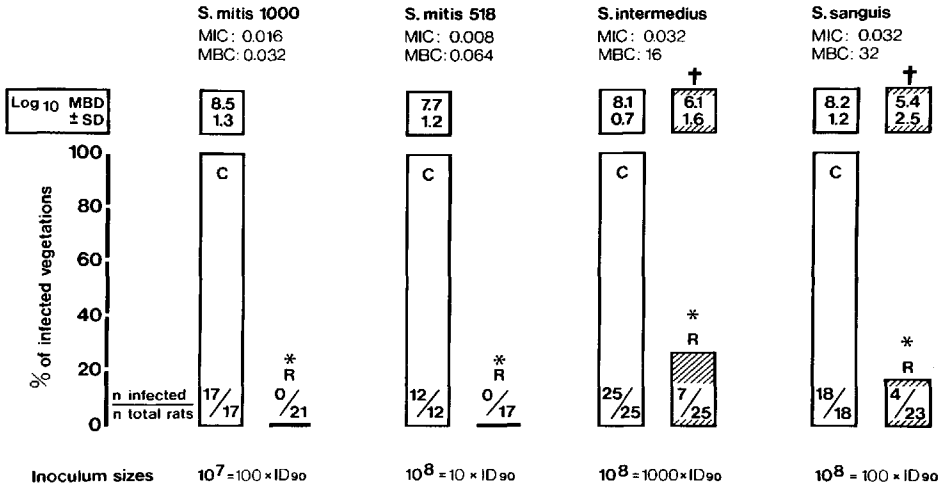

Figure 2. Incidence of endocarditis (number of rats with endocarditis/total number of rats challenged with each strain) in control (*C*) rats and in rats given single-dose rifampin prophylaxis (*R*) 4 hr before challenge with four strains of viridans streptococci. The inoculum size of each strain used for challenge was 10–1,000 times the ID_{90}. The MICs and MBCs of rifampin are indicated for each strain. The mean (± SD) bacterial densities (*MBD*) recovered from vegetations (expressed in cfu/g of vegetation) are indicated for each group. To compare the incidence of endocarditis in control rats to that in rats given rifampin prophylaxis, we calculated *P* values by χ^2 with Yates' correction; * = $P < .001$. To compare MBDs in controls to those in rats given rifampin prophylaxis, we calculated *P* values by unpaired Student's *t* test; † = $P < .003$.

phylaxis harbored streptococci with decreased in vitro susceptibility to rifampin.

Discussion

Experiments in both rabbits and rats have shown that when animals are challenged with organisms poorly susceptible to antibiotic killing, successful prophylaxis conferred by single doses of antibiotics is limited to the ID_{90} and fails when the animals are challenged with inocula higher than the ID_{90} [3, 6]. In the latter condition, endocarditis can be prevented only by prolonged levels of antibiotics, such as are achievable after multiple doses [18; R. M., P. Francioli, M. P. G, unpublished observations]. Because most strains of viridans streptococci appear to be poorly susceptible to the bactericidal action of cell wall-active antibiotics [4, 5], drugs providing high and prolonged serum antibiotic activity after a single dose should be investigated for preventing experimental bacterial endocarditis after challenge with heavy loads of bacteria.

The present results show that single-dose rifampin, which produces SIAs for >12 hr for all four strains tested, provided successful prophylaxis against very high bacterial inocula, irrespective of whether the organisms used for challenge were killed rapidly by the drug.

Against challenge with both *S. mitis* 1000 and *S. mitis* 518 (the two strains highly susceptible to killing by rifampin, as indicated by the high serum bactericidal levels in vivo and by time-kill curves in vitro), it is likely that protection was provided through bacterial killing. Previous experiments in rats [3] have shown that when bacteria used for challenge are killed rapidly enough in vitro that the intravenous bacterial inoculum is likely to be killed upon exposure to serum bactericidal levels in vivo, successful prophylaxis is independent of the inoculum size.

Against *S. sanguis* and *S. intermedius*, rapid bacterial killing could not account for successful prophylaxis because neither the serum bactericidal activity nor the in vitro exposure for 24 hr of both strains to peak concentrations of rifampin (10 μg/ml) indicated a significant reduction in bacterial numbers. In fact, it was established in vitro that this lack of bacterial killing was due to the selection of rifampin-resistant variants, a phenomenon observed in only one of 23 rats infected with 10^8 cfu of *S. sanguis* and given rifampin prophylaxis and in none of 25 rats infected with 10^8 cfu of *S. intermedius* and given rifampin prophylaxis.

In the absence of bacterial killing, one mechanism of successful endocarditis prophylaxis has been postulated to be antibiotic-mediated decreased adherence of organisms to damaged valvular endothelium [2, 17, 19]. By using an in vitro assay system simulating damaged valvular endothelium, Scheld et al. [17], as well as others [19], have shown that various cell wall–active antibiotics prevented the adherence of *S. sanguis* to platelet-fibrin matrices. Rifampin did not, however, influence the in vitro adhesion ratio of *S. sanguis* in those experiments, nor did it in the present experiments, when concentrations of rifampin >156 times the MICs of the two strains were used. Thus, the reduction of bacterial adherence by rifampin prophylaxis was not a likely mechanism operating to prevent endocarditis due to *S. intermedius* and *S. sanguis* in the present experiments.

Recent observations [18] have provided evidence that in the absence of rapid bacterial killing, the inhibition of growth of bacteria attached to the vegetations after intravenous challenge may be an important mechanism of protection after antibiotic prophylaxis, by permitting release of the bacteria that had colonized the vegetations. The longer the duration of growth inhibition, the greater the likelihood of successful prophylaxis after challenge with inocula exceeding the ID_{90} [18]. In the present experiments, single-dose rifampin provided serum levels above the MIC for both *S. intermedius* and *S. sanguis* for at least 12 hr. Moreover, it is likely that rifampin provided growth inhibition for a longer duration than the mere period of circulating rifampin levels: Bacterial numbers in vegetations from prophylaxis failures seven days after administration of rifampin were significantly lower than in controls, a result suggesting a prolonged in vivo suppression of bacterial growth. A similar phenomenon has been observed after amoxicillin prophylaxis of streptococcus endocarditis [3] and after rifampin prophylaxis of a foreign-body infection due to *Staphylococcus aureus* [20]. Moreover, a prolonged in vitro postantibiotic effect has been demonstrated for rifampin-exposed *S. aureus* [21].

The rapid development of resistant variants after administration of rifampin is a major concern. The lack of in vitro bactericidal activity of rifampin against *S. sanguis* and *S. intermedius* was due to the emergence of rifampin-resistant subpopulations after a long exposure to high concentrations of rifampin. In only one instance in vivo, however, could we detect moderately resistant *S. sanguis* after rifampin prophylaxis, a phenomenon that is not significant when one considers the total of 48 rats that received rifampin before challenge with high bacterial inocula of *S. sanguis* and *S. intermedius*. The inability of the in vitro observation to predict the in vivo outcome of prophylaxis might be explained by the fact that in vivo, both rifampin levels and duration of antibiotic exposure were less pronounced than in the in vitro experiments. It might be hypothesized that, in vivo, rifampin inhibited the growth of (or slowly killed) the susceptible subpopulation (i.e., the population with low MICs) of *S. intermedius* and *S. sanguis* and thus accounted for successful prophylaxis without selection of resistant variants.

In studies by others [14], the emergence of resistant strains was not observed in vivo when single-dose rifampin was used for prophylaxis of experimental *Staphylococcus epidermidis* endocarditis, nor was it observed after short-term treatment of experimental staphylococcus osteomyelitis (O. Zak, personal communication). In contrast, the emergence of rifampin resistance has been detected in experimental models when high numbers of bacteria were exposed to rifampin during prolonged periods of time [22–24]. Although these latter observations do not support the use of the prolonged administration of rifampin as a single therapeutic agent, short-term, single-dose rifampin prophylaxis might retain full effectiveness.

In conclusion, single-dose rifampin prophylaxis of experimental streptococcus endocarditis extended the protection to bacterial inocula exceeding, by far, the ID_{90} of the test strains. The protection was independent of whether or not the organisms were susceptible to bacterial killing by rifampin. Thus, rifampin was the first prophylactic agent that provided a wide margin of safety against experimental streptococcus endocarditis when given as a single dose.

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