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Efficacy of Temafloxacin in Experimental *Streptococcus adjacens* Endocarditis and Autoradiographic Diffusion Pattern of [^{14}C]Temafloxacin in Cardiac Vegetations

ANNE-CLAUDE CREMIEUX,[1]* AZZAM SALEH-MGHIR,[1] JEAN-MARIE VALLOIS,[1] BERNARD MAZIERE,[2] MARTINE MUFFAT-JOLY,[1] CATHERINE DEVINE,[3] ANNE BOUVET,[3] JEAN-JACQUES POCIDALO,[1] AND CLAUDE CARBON[1]

Hôpital Bichat-Claude Bernard, Institut National de la Santé et de la Recherche Médicale, Unité 13,[1] and Département de Microbiologie, UFR Broussais-Hôtel Dieu,[3] Paris, and Service Hospitalier Frédéric Joliot, Commissariat à l'Énergie Atomique, Orsay,[2] France

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Temafloxacin, a new fluoroquinolone, alone or in combination with tobramycin, was compared with penicillin, tobramycin, and their combination in the therapy of rabbits with endocarditis caused by *Streptococcus adjacens* GaD^T, a new species of nutritionally variant streptococci. Animals were injected intramuscularly for 4 days with temafloxacin (50 mg/kg of body weight twice daily [b.i.d.]) alone or combined with tobramycin (12 mg/kg once daily), with procaine penicillin (150,000 U/kg b.i.d.) alone or combined with tobramycin (12 mg/kg once daily), or with tobramycin (12 mg/kg once daily) alone. Another group of animals was treated with a higher dose of temafloxacin (100 mg/kg b.i.d.). Temafloxacin, penicillin, and tobramycin MICs and MBCs were 1 and 2, 0.015 and 1, and 8 and 16 μg/ml, respectively. Time-kill curves showed that the addition of tobramycin to penicillin or temafloxacin increased the killing rate. In vivo, treatment with temafloxacin (50 and 100 mg/kg b.i.d.) alone reduced the bacterial counts in vegetations (3.9 ± 0.9 and 3.1 ± 0.8 $\log_{10}$ CFU/g of vegetation) compared with those in the vegetations of control animals (7.5 ± 0.9 $\log_{10}$ CFU/g of vegetation). This result was similar to that obtained with penicillin alone (4.5 ± 0.8 $\log_{10}$ CFU/g of vegetation). The combination of temafloxacin (50 mg/kg) and tobramycin was as effective as penicillin plus tobramycin (2.5 ± 0.3 versus 2.3 ± 0.4 $\log_{10}$ CFU/g of vegetation, respectively). The autoradiographic pattern of [^{14}C]temafloxacin diffusion into infected cardiac vegetations was studied. Thirty minutes after the end of infusion of 250 μCi of [^{14}C]temafloxacin, the [^{14}C]temafloxacin was homogeneously distributed throughout the vegetations. These data support further evaluation of quinolones in experimental endocarditis.

Nutritionally variant streptococci (NVS) include two individual species, *Streptococcus defectivus* and *Streptococcus adjacens* (3), that are responsible for 5% of the cases of human bacterial endocarditis (19) and have a higher rate of complications than endocarditis caused by other viridans group streptococci (4). As for the other viridans group streptococci, a regimen combining penicillin plus an aminoglycoside is usually recommended for treatment of NVS endocarditis (1). Because of the high rates of complications and relapses, some investigators recommend 4 weeks of combination therapy for NVS endocarditis (10a). Vancomycin is usually considered the only safe and effective alternative therapy in the case of intolerance to β-lactams (2). However, vancomycin used in combination with an aminoglycoside can enhance the toxicity of the latter compound, especially if long-term therapy is needed (23). Temafloxacin is a new fluoroquinolone with a broad spectrum of activity. In vitro, temafloxacin was reported to be two- to eightfold more potent than ciprofloxacin or ofloxacin against various strains of viridans group streptococci, with MICs comparable to those of ceftriaxone (7a), and thus deserves to be evaluated as a potential alternative to the treatment of endocarditis when penicillin cannot be used. Although temafloxacin's concentrations have been measured in fibrin clots (22a), the pattern of diffusion of temafloxacin into fibrin vegetations is not known. The aims of this study were to evaluate, in an experimental model of endocarditis, the in vivo efficacy of temafloxacin alone and in combination with tobramycin when given at doses which result in concentrations in plasma similar to those obtained in humans and to compare these regimens with penicillin alone and combined with tobramycin. Tobramycin was preferred over another aminoglycoside because its diffusion pattern in vegetations has been described previously (8) and its in vitro activity is similar to that of gentamicin, which has been studied extensively (2). Furthermore, as shown in previous experiments (20a), in combination with penicillin, a once-daily dose of tobramycin is as effective as the same daily dosage administered three times daily (20a), and so the once-daily regimen was chosen because it is easier to administer. Finally, the distribution pattern of an antibiotic throughout infected cardiac vegetations may represent an important factor conditioning in vivo activity, and because it varies from one compound to another (8, 9), we studied the diffusion of [^{14}C]temafloxacin inside vegetations by autoradiography.

(This work was presented in part at the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy [20b].)

MATERIALS AND METHODS

Test strain. *S. adjacens* GaD^T (ATCC 49175^T) was used in this study. It was isolated from the cardiac valves of a patient with endocarditis who underwent surgery after 2 weeks of treatment with penicillin G and streptomycin (7).

* Corresponding author.

This strain was used to induce endocarditis in rabbits to study both the antibiotic therapy already prescribed to patients (2, 15) and the diffusion of new molecules into large vegetations (8, 9). The strain was grown at 37°C in a chemically defined medium enriched with 2% Todd-Hewitt dialysate (CDMT) (5).

In vitro antibiotic susceptibility tests. MICs and MBCs were determined in triplicate by the tube macrodilution method in CDMT containing a lower concentration of cysteine (5 mg/liter; (L-Cys CDMT) to minimize inactivation of penicillin (17). The inocula were diluted from log-phase cultures to obtain a final concentration of 10^5 CFU/ml. The antibiotics tested were penicillin G (Specia, Paris, France), tobramycin (Eli Lilly, Saint-Cloud, France), and temafloxacin (Abbott, Rungis, France). The MIC was defined as the lowest concentration of antibiotic that prevented turbidity in the test tube after 24 h of incubation, and the MBC was defined as the lowest concentration of antibiotic that reduced the number of organisms by 99.9% of the original number. MBCs were determined by plating, after 24 h of incubation, 0.1 ml from each clear broth tube onto 5% sheep blood Columbia agar (Bio-Mérieux, Marcy l'Etoile, France) enriched with pyridoxal hydrochloride (100 μg/ml), and the plates were incubated for 48 h. Tolerance was defined as an MBC/MIC ratio of greater than 32 (20).

Time-kill curves were plotted with log-phase inocula of 10^7 CFU/ml in L-Cys CDMT containing penicillin G (20 μg/ml), temafloxacin (8 μg/ml), and tobramycin (18 μg/ml) concentrations similar to the peak levels obtained in the sera of treated animals. In addition, temafloxacin was used at a higher concentration (25 μg/ml) in order to determine a possible concentration effect in vitro. Tobramycin was also tested at the subinhibitory concentration of 2 μg/ml (1/4 the MIC) in order to visualize a synergistic effect in combination with penicillin G or temafloxacin.

Samples were removed from the tubes after 0, 3, 6, and 24 h and were subjected to 10-fold serial dilutions in sterile saline, with 0.1 ml of each dilution being seeded on enriched agar plates, which were incubated for 48 h. Penicillinase was added to agar for samples containing penicillin. Each experiment was performed twice in duplicate and included controls of growth without antibiotic. In vitro synergy was defined as a 100-fold increase of killing by the combination over that obtained with the single most effective agent and a 1,000-fold decrease of the bacterial count compared with that of the inoculum. The minimal detectable number of CFU per milliliter was 10.

Experimental endocarditis. A modified version of the method of Perlman and Freedman (18) was used to induce endocarditis in female New Zealand White rabbits, each weighing between 2 and 3 kg. As described previously (2), a polyethylene catheter was inserted through the right carotid artery into the left ventricular cavity and was left in place throughout the experiment. Twenty-four hours after placement of the catheter (day 1), animals were inoculated via the marginal ear vein with 10^8 CFU of strain GaD^T in 1 ml of saline. This resulted in endocarditis in 100% of the inoculees, when the catheter was correctly placed, as demonstrated by positive blood cultures on day 7 and by the presence of infected vegetations on the aortic valves at the time of sacrifice. Blood cultures were performed as described previously (2).

Therapeutic studies. (i) Treatment and evaluation of therapy. Six days after infection (day 7), the following intramuscular (i.m.) injections were started: procaine penicillin (150,000 U/kg of body weight twice daily [b.i.d.], i.m.), temafloxacin (50 mg/kg b.i.d., i.m.), and tobramycin (12 mg/kg once daily i.m.) alone or in combination with either procaine penicillin or temafloxacin. These doses were chosen to obtain concentrations in plasma comparable to those achieved in humans. Another group of rabbits was treated with temafloxacin (100 mg/kg b.i.d., i.m.) in order to assess a potential in vivo dose effect. Each treatment regimen was administered for 4 days. Animals were killed on day 11, 12 h after the last dose of penicillin and temafloxacin or 24 h after the last dose of tobramycin. Untreated control rabbits were sacrificed at the time corresponding to the end of therapy (day 11); one of these animals died on day 8.

At the time of sacrifice, all vegetations were excised, weighed separately, homogenized in 0.5 ml of saline, and quantitatively cultured on pyridoxal-enriched blood agar plates at 37°C for 48 h. The lowest detectable bacterial counts ranged from 2 to 3.5 $\log_{10}$ CFU/g of vegetation, depending on the size of the vegetation. The vegetation was considered sterile when the culture showed no growth after incubation for 48 h at 37°C, and the number of CFU was recorded as the lowest detectable bacterial count. Drug carryover was avoided by serial dilutions and spreading of the subculture (50 μl) on agar plates or by adding penicillinase to agar for samples containing penicillin.

(ii) Antibiotic levels in serum. Blood was drawn from the ear vein 1 h after the injection of antibiotic on day 10 and at the time of sacrifice for determination of peak and trough concentrations, respectively, and was stored at −70°C. Penicillin concentrations were measured by agar disk diffusion by using *Bacillus subtilis* ATCC 6633 as the test strain. Temafloxacin and tobramycin concentrations were measured by high-performance liquid chromatography and a radioenzyme assay, respectively. The lower limits of detection were 0.1 μg/ml for penicillin, 0.015 μg/ml for temafloxacin, and 0.015 μg/ml for tobramycin; and assay reproducibilities were, ±7, ±5, and ±6%, respectively.

(iii) Statistics. Bacterial densities in vegetations within the experimental groups were compared by an analysis of variance; this was followed by the Scheffe test for multiple comparisons. Results are expressed as means ± standard deviations. The percentage of rabbits with sterile vegetations was compared by the χ^2 test by using Yates' continuity correction. A P value of <0.05 was considered significant.

Quantitative autoradiography of [^{14}C]temafloxacin diffusion into infected vegetations. Quantitative autoradiography of antibiotic diffusion into infected vegetations has recently been described in detail (8). Briefly, two infected animals with large vegetations, as assessed by two-dimensional echography, were selected for the study. On day 11, 250 μCi of [^{14}C]temafloxacin (71.9 mCi/mg; kindly provided by Abbott Laboratories, Rungis, France) in 10 ml was injected intravenously over 30 min. Determination of antibiotic concentrations in plasma and vegetations by liquid scintillation counting and quantitative autoradiography of vegetations was performed 30 min after the end of the infusion. X-ray films exposed to the vegetation sections for 6 weeks were developed, and autoradiographic images were quantified. A total of three vegetations were studied.

RESULTS

In vitro studies. The MICs and MBCs of temafloxacin, penicillin, and tobramycin were 1 and 2, 0.015 and 1, and 8 and 16 μg/ml, respectively, indicating susceptibility to temafloxacin, tolerance to penicillin, and low-level resistance to tobramycin.

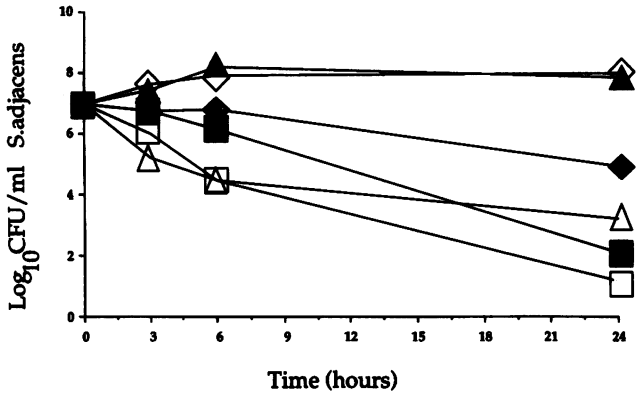

FIG. 1. Time-kill study comparing the effects of antibiotics on *S. adjacens* GaD^T. Symbols: ▲, control; ◆, penicillin G at 20 μg/ml; △, temafloxacin at 8 μg/ml; ◇, tobramycin at 2 μg/ml; ■, penicillin at 20 μg/ml plus tobramycin at 2 μg/ml; □, temafloxacin at 8 μg/ml plus tobramycin at 2 μg/ml.

The decreases in bacterial counts, expressed in $\log_{10}$ CFUs per milliliter, at 3, 6, and 24 h after exposure to the drug were 0.2, 0.1, and 2.0, respectively, for penicillin (20 μg/ml) (Fig. 1); 1.7, 2.4, and 3.8, respectively, for temafloxacin (8 μg/ml) (Fig. 1); and 1.5, 3.5, and 4.5, respectively, for tobramycin (18 μg/ml) (data not shown). The temafloxacin killing rate was slightly increased (4.6 $\log_{10}$ decrease at 24 h) in the presence of the higher concentration (25 μg/ml) (data not shown). Combinations of a subinhibitory concentration of tobramycin (2 μg/ml) with penicillin or temafloxacin increased the killing rate (4.7 and 5.3 $\log_{10}$ decrease at 24 h with both combinations, respectively) (Fig. 1) but were not synergistic as defined in Materials and Methods.

Therapeutic studies. Antibiotic concentrations in plasma are given in Table 1. Temafloxacin and penicillin levels were greater than the MICs for the infecting strain for the entire therapeutic interval. The peak tobramycin level was fourfold its MIC. In all treated groups, the mean bacterial counts in vegetations were significantly reduced compared with those in control animals ($P < 0.001$) (Table 2). The mean bacterial counts in vegetations of the temafloxacin, penicillin, and tobramycin single-drug regimen groups were comparable ($P > 0.05$). The efficacy of a higher dose of temafloxacin (100 mg/kg b.i.d.) was not significantly different from that of the 50-mg/kg dose (3.1 ± 0.8 versus 3.9 ± 0.9 $\log_{10}$ CFU/g). The combination of tobramycin and penicillin significantly reduced the bacterial counts in vegetations compared with the reduction in bacterial counts after treatment with penicillin alone ($P < 0.05$) but not after that with tobramycin alone.

TABLE 1. Antibiotic concentrations measured in serum after 4 days of treatment given i.m.

Antibiotic	Concn (μg/ml)	
	Peak[a]	Trough[b]
Temafloxacin, 50 mg/kg b.i.d.	7.4 ± 2.8	2.2 ± 0.9
Temafloxacin, 100 mg/kg b.i.d.	19.3 ± 1.6	14.2 ± 2.7
Procaine penicillin, 150,000 U/kg b.i.d.	22.2 ± 11.3	0.7 ± 0.3
Tobramycin, 12 mg/kg once daily	17.5 ± 14.4	0.4 ± 0.4

[a] Samples obtained 1 h after the last injection of antibiotic.
[b] Samples obtained 12 h after the last dose of penicillin and temafloxacin and 24 h after the last dose of tobramycin.

TABLE 2. Results of antibiotic treatment of experimental *S. adjacens* endocarditis

Treatment[a]	No. of rabbits	No. of rabbits with sterile vegetations	Mean ± SD $\log_{10}$ CFU/g
Control	7	0	7.5 ± 0.9
Procaine penicillin	6	0	4.5 ± 0.8[b]
Temafloxacin	8	1	3.9 ± 0.9[b]
Tobramycin	7	2	4.2 ± 1.5[b]
Procaine penicillin-tobramycin	6	6	2.6 ± 0.4[b,c]
Temafloxacin-tobramycin	7	6	2.3 ± 0.4[b,c,d]

[a] Rabbits were treated i.m. for 4 days with procaine penicillin (150,000 U/kg b.i.d), temafloxacin (50 or 100 mg/kg b.i.d.), tobramycin (12 mg/kg once daily) or procaine pencillin-tobramycin or temafloxacin-tobramycin combinations.
[b] Significantly different from controls ($P < 0.001$).
[c] Significantly different from penicillin alone ($P < 0.05$).
[d] Significantly different from tobramycin alone ($P < 0.05$).

The combination of temafloxacin and tobramycin did not significantly reduce the bacterial counts in vegetations compared with that after treatment with temafloxacin alone at either dose ($P > 0.005$). However, when the percentage of rabbits with sterile vegetations in the treated groups was considered, both combination regimens appeared to be more effective than the single-drug regimen ($P < 0.05$ *to* 0.01) except for the comparison of temafloxacin-tobramycin and tobramycin alone. Both combination regimens gave similar in vivo results when either the mean bacterial counts in vegetations or the numbers of rabbits with sterile vegetations were considered. No temafloxacin-resistant strain emerged in the vegetations of treated animals in any groups.

[^{14}C]temafloxacin diffusion into cardiac vegetations. Thirty minutes after a single infusion of 250 μCi of [^{14}C]temafloxacin, the mean for three vegetation/blood radioactivity ratio determinations was 2.16 ± 0.35, and the mean for three vegetation/cardiac tissue radioactivity ratio determinations was 1.12 ± 0.18.

Temafloxacin was homogeneously distributed throughout all three vegetations examined. An example is shown in Fig. 2. The concentrations of labeled antibiotic (nanocuries per gram) in the different parts of the vegetation are given in the diagram corresponding to the autoradiograph.

DISCUSSION

In our model of endocarditis, temafloxacin appeared to be equivalent to penicillin after 4 days of therapy. Temafloxacin levels in the plasma of rabbits were comparable to those obtained in humans after a 1,000-mg single oral dose (14), a dose higher than that recommended for treatment of other infections (600 mg). However, in light of the severity of endocarditis, such a high dose can be justified. Most of the rabbits treated with penicillin or temafloxacin alone had infected vegetations at the time of sacrifice. Despite the low penicillin MICs, the response to penicillin therapy varies considerably (4) in NVS endocarditis in humans, and combination therapy with an aminoglycoside is usually recommended for 2 to 4 weeks (1).

The results of the in vitro time-kill studies correlated inconsistently with those of the in vivo experiments. This discordance has been noted previously (2). Indeed, despite a more rapid killing activity in vitro, temafloxacin alone did not appear to be more effective than penicillin alone in vivo after 4 days of therapy. As shown by our results, this

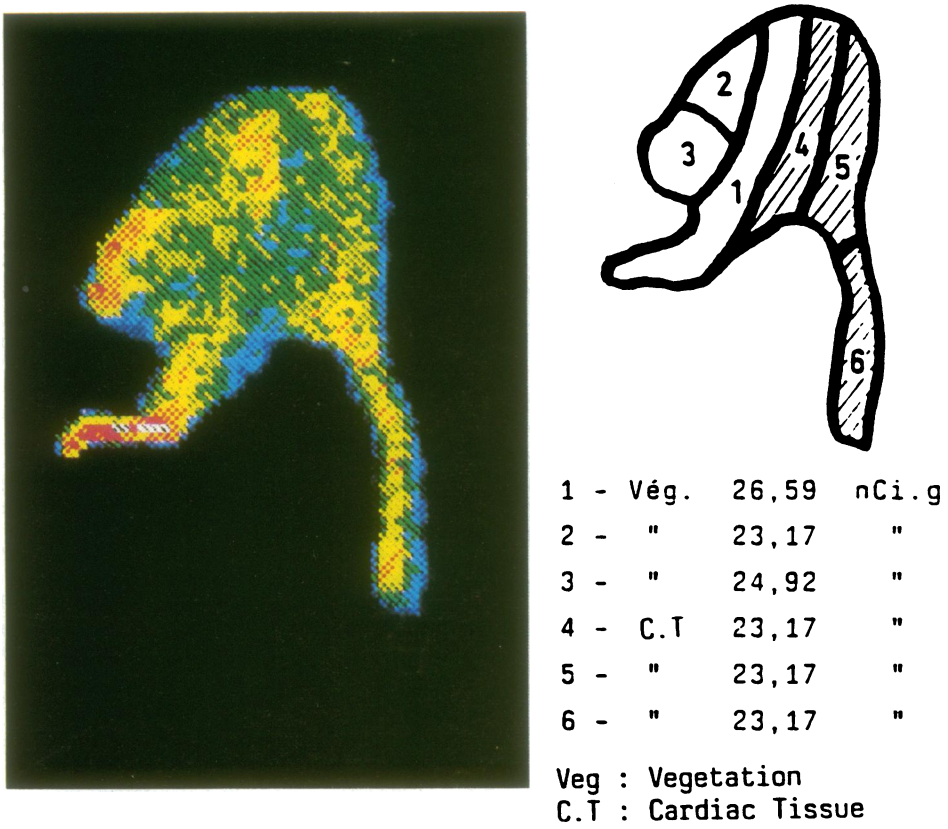

FIG. 2. Quantitative autoradiograph of a vegetation and adjacent cardiac tissue taken from a [^{14}C]temafloxacin-treated rabbit 30 min after the end of the intravenous infusion. The values given in the vegetation (Veg) and cardiac tissue (C.T.) diagram next to the autoradiograph refer to the radioactivity (nanocuries per gram) of labeled antibiotic for each zone indicated in the autoradiograph. Magnification, ×15.

observation cannot be explained by an impaired diffusion into vegetations. A serum effect seems unlikely, since this effect is observed mostly with antibiotics with high levels of serum protein binding. No serum effect was seen with temafloxacin on *Staphylococcus aureus* (22). Moreover, this effect is usually circumvented by increasing the dose in order to increase the percentage of free antibiotic in the serum. In this study, the in vitro killing rate of temafloxacin was not markedly affected by a tripling of its concentration. An increase in the temafloxacin dose from 50 to 100 mg/kg in vivo did not modify the therapeutic results after 4 days of therapy. With this latter dose, which is clinically unrealistic, we observed high trough concentrations, suggesting a cumulative effect. The discrepancy between the results of in vitro and in vivo experiments might be related to differences in the growth conditions of these fastidious organisms. The medium used in the present study, CDMT, has been shown to be suitable for balanced growth, but it does not simulate the growth conditions in vegetations. Frehel et al. (11) have shown that, in the vegetation, the ultrastructure of the organism is modified and that the observed modifications suggested impaired growth conditions. The influence of the growth rate on the susceptibilities of these streptococci to fluoroquinolones as well as the presence in vivo of an exopolysaccharide coat on the organisms in the vegetations (11) remains to be clarified, but it could account for the differences noted between in vitro and in vivo temafloxacin activities.

The activities of tobramycin in vitro and in vivo were similar to those of temafloxacin. Peak and trough tobramycin levels in serum were the same as those obtained with a once-daily aminoglycoside dosage in humans, which is often recommended (12). Although aminoglycosides alone are not recommended for treatment of streptococcal endocarditis, gentamicin has been shown to exhibit in vivo activity against enterococci, even though resistance has been observed in vitro, as measured by conventional assays to determine MICs and MBCs (16 and 32 μg/ml, respectively, for the *Streptococcus faecalis* strain tested) (21).

The combination of tobramycin and penicillin significantly enhanced the efficacy of penicillin alone. This result is in agreement with those of Carey et al. (6) and our previous study (2), which demonstrated an in vivo synergism between penicillin and other aminoglycosides. The addition of tobramycin to temafloxacin also decreased the $\log_{10}$ CFU per gram of vegetation compared with that of temafloxacin alone, although the difference was not significant. Nevertheless, considering the number of rabbits with sterile vegetations, the combination groups were significantly more effective than the single-drug regimens, except for temafloxacin-tobramycin compared with tobramycin alone. The combination of temafloxacin and tobramycin was as effective as penicillin plus tobramycin, which was taken as the reference regimen for NVS endocarditis in this study, and six of seven rabbits had sterile vegetations at the end of treatment. This result is in agreement with time-kill curves.

In the present study, we showed that [^{14}C]temafloxacin penetrated into and homogeneously diffused throughout the

vegetations. Although it seems logical to hypothesize that, to sterilize a vegetation, the antibiotic should be able to diffuse throughout the infective lesion, the predictive value of the autoradiographic diffusion pattern of antibiotics into vegetation for therapeutic efficacy is still not known. The concentration gradient between the periphery and the core of the vegetation observed with [^{14}C]ceftriaxone (9) and, to a lesser degree, with [^{14}C]penicillin (8) could explain the need for local concentrations to significantly exceed the MIC and/or MBC for the offending organism (16), as assessed by determination of the drug concentration in a homogenate of the vegetation. The failure of teicoplanin to diffuse into the core of the vegetation could be one explanation for the high rates of failure observed with this antibiotic in the therapy of human staphylococcal endocarditis (13). However, this would be difficult to demonstrate in vivo by a comparative therapeutic study of the two antibiotics with different patterns of diffusion, because other factors, such as the killing rate of the antibiotic, the possible inactivation of the antibiotic by local physicochemical conditions, and the metabolic state of the bacteria, must be taken into account. In addition to the distribution pattern, it is important to consider the concentration of antibiotic in contact with the bacteria. The [^{14}C]temafloxacin vegetation/blood concentration ratio 30 min after the end of the infusion is about 2, a value comparable to that obtained 30 min after the end of the infusion with [^{3}H]tobramycin, which also diffuses homogeneously. These findings must be interpreted with caution because they are values obtained at only one point in time. Pharmacokinetic analyses at different sampling times after drug injection into animals are hardly feasible by our autoradiographic method for economic reasons. However, for antibiotics that diffuse homogeneously, the concentrations in homogenates determined by usual methods may reflect the concentrations throughout the lesion. Knowing these limitations, the demonstration of a homogeneous autoradiographic pattern of distribution of [^{14}C]temafloxacin suggests that the fibrin matrix, which represents the major component of vegetations (10), does not impede the diffusion of temafloxacin toward the bacteria inside vegetations and is compatible to our therapeutic results as well as those obtained in experimental staphylococcal endocarditis (22). Similar homogeneous diffusion patterns have been found with two others quinolones, pefloxacin and sparfloxacin (9a). It must be emphasized that temafloxacin was tested in vivo against only one strain of *S. adjacens*. However, in light of the need for an alternative therapy in the case of β-lactam intolerance, our therapeutic results showing that temafloxacin in combination with tobramycin is as effective as the combination of penicillin and tobramycin and the demonstration of an homogeneous diffusion of [^{14}C]temafloxacin into fibrin vegetations support further experimental evaluation of temafloxacin or other new quinolones with in vitro antistreptococcal activity in endocarditis caused by other strains.

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