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In Vivo Activities and Penetration of the Two Components of the Streptogramin RP 59500 in Cardiac Vegetations of Experimental Endocarditis

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We evaluated the in vivo activity and the diffusion of radiolabelled RP 57669 (RPI) and RP 54476 (RPII), the two components of the injectable streptogramin RP 59500, alone or in combination, in aortic vegetations from experimental endocarditis in rabbits. RPI and RPII demonstrated in vitro bacteriostatic and bactericidal synergy against a clinical strain of *Staphylococcus aureus* resistant to methicillin and susceptible to erythromycin. In experimental staphylococcal endocarditis, RP 59500 was as effective as vancomycin and significantly more effective than RPI ($P < 0.01$) and RPII ($P < 0.05$). Autoradiography studies showed different patterns of distribution into cardiac vegetations infected with *Streptococcus sanguis* for [^{14}C]RPI and [^{14}C]RPII. [^{14}C]RPI was homogeneously distributed throughout the vegetations whereas [^{14}C]RPII showed a decreasing gradient of concentration between the periphery and the core of the vegetation, with an approximately 2:1 ratio. [^{14}C]RPI diffused approximately 2 to 4 times more than [^{14}C]RPII into the core of the vegetations. Since the injected ratio of RPI and RPII is 30:70 in RP 59500, the actual RPI:RPII ratio in the core of the vegetation may range from 0.8 to 1.7, a ratio which remains compatible with the in vivo synergism demonstrated between the two components.

Methicillin-resistant *Staphylococcus aureus* is a major cause of nosocomial infection, and vancomycin remains the standard antimicrobial agent for the therapy of systemic infections due to methicillin-resistant *S. aureus*. Few alternative therapies to vancomycin are available since most of these strains are also resistant to aminoglycosides, fluoroquinolones, and rifampin in many countries (13, 14).

RP 59500, a new semisynthetic injectable streptogramin, is a combination of two semisynthetic compounds, combined in a 30:70 ratio: RP 57669 (referred to as RPI) is a peptide macrolactone classified as a streptogramin B, and RP 54476 (referred to as RPII) is a polyunsaturated macrolactone classified as a streptogramin A. In vitro, RP 59500 is active against gram-positive cocci, and particularly *S. aureus*, with a MIC for 90% of strains tested of ≤1 μg/ml for methicillin-susceptible, methicillin-resistant, and multiple-resistant *S. aureus* (1). The in vitro activity of RP 59500 is due to the synergistic activity of RPI and RPII (3).

In order to study the potential synergism in vivo between RPI and RPII, we evaluated the pharmacodynamic and pharmacokinetic interactions of the two components of RP 59500 in experimental endocarditis in rabbits, which is a model of severe infection. We therefore (i) evaluated the in vivo activity of RPI and RPII, alone or in combination, in an experimental endocarditis due to methicillin-resistant *S. aureus* and (ii) studied by autoradiography the diffusion of [^{14}C]RPI and [^{14}C]RPII, alone or in combination with cold RPII and RPI, respectively, into the vegetations in an experimental endocarditis due to *Streptococcus sanguis*, a model of subacute endocarditis that provided large vegetations suitable for the autoradiography study.

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MATERIALS AND METHODS

In vitro studies. (i) Organisms. *S. aureus* HM1054 was isolated from the blood of a patient with septicemia. This strain was resistant to methicillin and susceptible to erythromycin and was used to study the in vitro and in vivo activities of antimicrobial agents. *S. sanguis* I ATCC 10556 was used to produce experimental endocarditis for autoradiography studies.

(ii) Media and antibiotics. Mueller-Hinton broth (MHB) and Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) were used. All incubations were done at 37°C. Drugs were supplied by their manufacturers: vancomycin by Eli Lilly & Co. (Saint-Cloud, France) and cold and radiolabelled RPI and RPII and RP 59500 by Rhône-Poulenc Rorer (Vitry sur Seine, France).

(iii) In vitro susceptibility to antibiotics. The MICs and MBCs of vancomycin, RPI, RPII, and RP 59500 were determined by the macrodilution method, with an inoculum of 5×10^5 CFU/ml. The MIC was defined as the lowest concentration of antibiotic that prevented turbidity after 24 h of incubation. The MBC was defined as the lowest concentration of antimicrobial agent that killed at least 99.9% of the original inoculum (16).

(iv) Study of combined antimicrobial activity. Time-kill curves were used to test the bactericidal activities of vancomycin, RPI, and RPII, alone or in combination. Overnight cultures were diluted in glass tubes containing 10 ml of fresh MHB to yield an inoculum of 5×10^6 CFU/ml. The concentrations used were 4 μg/ml for vancomycin, 2 μg/ml for RPI and RPII, and 0.5 and 2 μg/ml for RP 59500. After 0, 3, 6, and

24 h of incubation at 37°C, serial dilutions of 0.1-ml samples were subcultured onto agar plates, by using a spiral plater (Spiral System Inc., Cincinnati, Ohio), and incubated for 24 h before CFU were counted. In preliminary experiments, antibiotic carryover was ruled out by plating samples of bacterial suspensions containing 10 to 10^3 CFU/ml in the presence or absence of antibiotics alone or in combination (16). Bactericidal activity was defined as an at least 3 $\log_{10}$ decrease in the original inoculum (16). Synergism was defined as a ≥100-fold increase in killing at 24 h with the combination of RPI and RPII in comparison with the most active single drug (7). The results were the means of two sets of experiments.

In vivo studies. For staphylococcal experimental endocarditis, investigations were performed in female New Zealand White rabbits (weight range, 2.2 to 2.8 kg). Aortic endocarditis was induced by insertion of a polyethylene catheter through the right carotid artery into the left ventricle to induce the formation of vegetations. Twenty-four hours after catheter insertion, each rabbit was inoculated by ear vein with 10^6 CFU of *S. aureus* (range, 10^6 to 4×10^6 CFU) in 1 ml of 0.9% NaCl. This inoculum produced endocarditis in all rabbits, with proper placement of the catheter. The catheter was left in place throughout the experiment. Within 2 days after bacterial inoculation, approximately 30% of the animals died from sepsis. Untreated rabbits were killed 48 h after bacterial inoculation and served as controls. The weight of the vegetations at that time ranged from 6 to 46 mg. Forty-eight hours after inoculation, animals were treated intramuscularly every 12 h for 4 days with one of the following regimens (each at 30 mg/kg of body weight): vancomycin, RPI, RPII, or RP 59500. The vancomycin regimen was chosen because it reproduced levels in serum comparable to those obtained in humans (10). The dose of RP 59500 was chosen because it produced peak levels in serum in the range of those obtained in humans (8) and because higher doses have been shown to be no more effective but toxic in rabbits (4). Given the fact that the 30-mg/kg dose of RP 59500 actually corresponded to 9 mg of RPI per kg and 21 mg of RPII per kg, doses of 30 mg of RPI and RPII per kg were used in order to reinforce the demonstration of a potential in vivo synergism.

Animals were killed by intravenous injection of pentobarbital. The heart was removed, and the chambers on the left side were examined to confirm vegetative endocarditis. Only animals with proper placement of the catheter and macroscopic evidence of vegetation at the time of sacrifice were included in the study. All vegetations from each rabbit were excised, pooled, and weighted. They were homogenized in 0.5 ml of sterile saline, and 0.1-ml portions were quantitatively subcultured onto agar plates for 24 h. Colony count results were expressed as $\log_{10}$ CFU per gram of vegetation.

Portions (0.1 ml) from the undiluted and the 1:10 suspension of vegetations from the animals treated with RP 59500 were plated onto agar plates containing a final concentration of 0.5, 1, and 2 μg of RP 59500 per ml and incubated for 48 h at 37°C in order to detect emergence of resistance.

Serum pharmacokinetic studies. (i) Samples. Vancomycin levels were determined in serum 1 h (peak) and 12 h (trough) after the last injection. Serum RP 59500 levels were determined in three infected rabbits after a single injection of 30 mg/kg intramuscularly. Two milliliters of blood was sampled at 15, 30, 45, 60, 90, 120, and 180 min after the injection, via a femoral catheter, and were immediately placed into a tube containing 0.5 ml of 0.25 N hydrochloric acid; the mixture was stirred strongly by hand and centrifuged (10 min, 1,500 × *g*). The upper phase was stored at −70°C. Three additional infected rabbits were killed 1 h after a single intramuscular injection of 30 mg of RP 59500 per kg, and vegetations and blood were sampled for the determination of RP 59500 concentrations.

TABLE 1. MICs and MBCs of antibiotics against *S. aureus* HM1054

Antibiotic	MIC (μg/ml)	MBC (μg/ml)
Vancomycin	1	≥16
RP 59500	0.5	0.5
RPI	8	128
RPII	4	64

(ii) Assays. Antibiotic concentrations were measured in serum and in vegetations by the agar diffusion method. Indicator organisms were *Micrococcus luteus* ATCC9341 and *Bacillus subtilis* ATCC6633 for RP 59500 and vancomycin, respectively. Medium no. 2 and medium no. 1 (Difco) were used for RP 59500 and vancomycin, respectively. The sensitivity of the assay was 0.1 and 0.5 μg/ml for RP 59500 and vancomycin, respectively.

Autoradiography studies. (i) Streptococcal endocarditis. Endocarditis was produced as described above. Animals were infected 24 h after placement of the catheter with 10^8 CFU of *S. sanguis*.

(ii) ^{14}C-antibiotic administration and assay. Eight days after bacterial challenge, radiolabelled antibiotics were injected intravenously in a volume of 10 ml of saline over a period of 30 min. Four different regimens of radiolabelled antibiotics were studied: two rabbits received [^{14}C]RPI (116 ± 26 μCi), one rabbit received [^{14}C]RPI (186 μCi) plus cold RPII, one rabbit received [^{14}C]RPII (144 μCi), and two rabbits received [^{14}C]RPII (195 ± 23 μCi) plus cold RPI. The ratio of concentrations between RPI and RPII was 30:70 for the regimens with both antibiotics, in order to reproduce the ratio in RP 59500. Blood and plasma samples were collected at the end of infusion and 30 min later. At that time, animals were killed and entire vegetations were excised; some vegetations were used for autoradiography, and others were used for measurement of antibiotic concentrations. Labelled antibiotic concentrations were determined in samples of plasma, blood, cardiac muscle, and vegetations by liquid scintillation counting, as previously described (5), and expressed as desintegrations per minute per gram. The results were expressed as the mean radioactivity concentration in vegetation per injected radioactivity and the ratios of mean concentrations of antibiotics in vegetation to those in plasma, blood, and cardiac tissue.

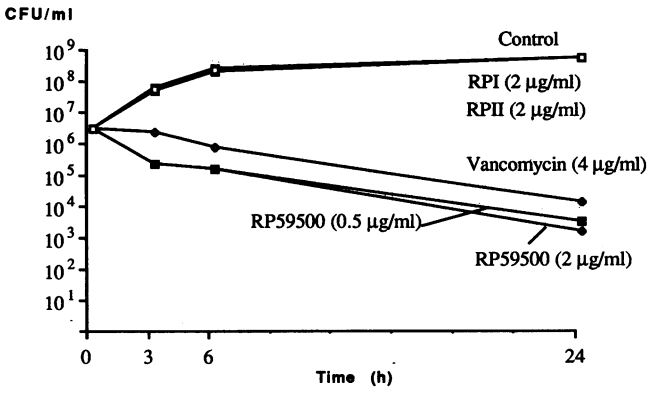

FIG. 1. In vitro-killing rates of *S. aureus* HM1054 incubated with vancomycin, RP 59500, RPI, or RPII in MHB.

TABLE 2. Results of different 4-day treatments (30 mg/kg intramuscularly twice a day) in rabbits infected with *S. aureus* HM1054

Treatment	No. of survivors/ no. of treated animals	Log_{10} CFU/g of vegetation (mean ± SD)
None (control)	—/8[a]	8.3 ± 1.1
Vancomycin	7/7	6.3 ± 1.2[b]
RP 59500	5/7	5.6 ± 1.1[c,d]
RPI	3/5	8.0 ± 0.4
RPII	0/5	8.1 ± 0.6

[a] —, control animals were sacrificed at the start of therapy.
[b] $P < 0.05$ (versus values for controls).
[c] $P < 0.01$ (versus values for controls).
[d] $P < 0.05$ (versus values for RPI and RPII).

(iii) Quantitative autoradiography. Frozen vegetation samples were cut on a cryostat, as previously described (5), thaw-mounted onto gelation-coated microscope slides, and freeze-dried at −25°C for 24 h. The sections were then apposed for 109 to 127 days against X-ray film in three-layer autoradiography cassettes. Included with each film were ^{14}C-labelled microscale standards. The exposed films were developed, fixed, washed, and dried at room temperature. Autoradiographs were quantified by using a videodensitometry digital-film analyzer. Digital sampling of transmitted light intensities through the autoradiographs was performed with 256 gray levels according to a 256 by 256 array size. The digitalized images were converted to radiotracer activities pixel by pixel, from the microscale standards.

(iv) Statistics. The bacterial concentrations in vegetations and the ratios of radiolabelled antibiotic concentrations from the various groups of animals were compared by an analysis of variance followed by the Scheffe test for multiple comparisons. A P value of <0.05 was considered significant. All results are expressed as means ± standard deviations.

RESULTS

In vitro data. MICs and MBCs for the different antibiotics tested are shown in Table 1. The MIC of RP 59500 was 16- and 8-fold less than those of RPI and RPII, respectively; the MBC of RP 59500 was 256- and 128-fold less than those of RPI and RPII, respectively. The in vitro synergism between RPI and RPII was confirmed by the killing curves (Fig. 1), with a reduction of more than 4 log_{10} CFU/ml at 24 h with RP 59500 at 0.5 μg/ml in comparison with RPI or RPII used at a concentration of 2 μg/ml. Increased concentrations of RP 59500 above the MBC did not significantly increase the bactericidal activity of the antibiotic at 24 h.

Antibiotic concentrations in serum. Vancomycin concentrations in serum were 27 ± 12 and 5 ± 3 μg/ml at peak (1 h) and trough (12 h), respectively. The peak concentration of RP 59500 in serum, obtained 15 min after the injection, was 3.08 ± 0.77 μg/ml. Mean levels of RP 59500 in serum were above the MIC and the MBC for the study strain for 3.9 h after a single injection. The mean ratio of the concentration of RP 59500 in vegetation to that in serum obtained 1 h after a single injection in each of three rabbits was 4.1.

Activity in staphylococcal endocarditis. The results of the different antibiotic regimens are shown in Table 2. Vancomycin ($P < 0.05$) and RP 59500 ($P < 0.01$) significantly reduced bacterial titers in vegetations at the end of therapy, whereas RPI and RPII alone were ineffective. RP 59500 was significantly more effective than RPI ($P < 0.01$) and RPII ($P < 0.05$). No vegetation from the animals treated with RP 59500 contained resistant clones. No animal had a sterile vegetation.

Concentration ratios of radiolabelled antibiotics. As shown in Table 3, [^{14}C]RPI, unlike [^{14}C]RPII, was more concentrated in vegetations than in cardiac muscle. The diffusion of [^{14}C]RPI and [^{14}C]RPII tended to be increased and more variable when coadministrated with cold RPII and cold RPI, respectively. More [^{14}C]RPI than [^{14}C]RPII tended to penetrate the vegetations; the mean vegetation/injected radioactivity, vegetation/plasma, vegetation/blood, and vegetation/cardiac tissue ratios of antibiotic were 2.5 to 3.7 times higher for [^{14}C]RPI than for [^{14}C]RPII, but the differences were not statistically significant. However, when coadministrated with cold RPII, vegetation/plasma, vegetation/blood, and vegetation/cardiac tissue ratios of [^{14}C]RPI were significantly higher ($P < 0.05$) than those of [^{14}C]RPII coadministrated with cold RPI.

Autoradiography. Quantitative autoradiographs of vegetations representative of [^{14}C]RPI and [^{14}C]RPII are shown in Fig. 2. The autoradiographic patterns were different for [^{14}C]RPI and [^{14}C]RPII. [^{14}C]RPI was distributed homogeneously throughout the vegetations; in contrast, [^{14}C]RPII exhibited a gradient of decreasing concentrations between the periphery and the core of the vegetation. However, [^{14}C]RPII reached the core of the vegetation, and the ratio of concentrations in the periphery and the core was approximately 2. The autoradiographic patterns of [^{14}C]RPI and [^{14}C]RPII were not modified when the antibiotics were administrated in combination with cold RPII and cold RPI, respectively: [^{14}C]RPI was still homogeneously distributed, and [^{14}C]RPII still exhibited a gradient of decreasing concentrations between the periphery and the core of the vegetation.

TABLE 3. Radioactively labelled antibiotic concentration in vegetation per injected radioactivity and vegetation/plasma, vegetation/blood, and vegetation/cardiac tissue ratios of [^{14}C]RPI and [^{14}C]RPII concentrations

Regimen (no. of animals)	Dose injected (μCi)	No. of vegetations	Radioactivity in vegetation/injected radioactivity[a]	Ratio of mean antibiotic radioactivity ± SD		
				Vegetation/plasma[b]	Vegetation/blood[b]	Vegetation/cardiac tissue[c]
[^{14}C]RPI (2)	116 ± 26	4	469 ± 151	1.83 ± 0.53	2.51 ± 0.73	4.97 ± 1.49
[^{14}C]RPI + cold RPII (1)	186	3	696 ± 283	3.08 ± 1.30[d]	3.81 ± 1.55[e]	7.99 ± 3.25[d]
[^{14}C]RPII (1)	144	3	186 ± 16	0.50 ± 0.04	0.68 ± 0.06	1.49 ± 0.13
[^{14}C]RPII + cold RPI (2)	195 ± 23	6	517 ± 275	0.79 ± 0.51	1.24 ± 0.72	1.07 ± 0.33

[a] (Disintegrations per minute per gram/disintegrations per minute), 10^{-6}.
[b] Ratio of disintegrations per gram/disintegrations per milliliter.
[c] Ratio of disintegrations per gram/disintegrations per gram.
[d] Significantly different from values obtained with [^{14}C]RPII plus cold RPI ($P < 0.01$).
[e] Significantly different from values obtained with [^{14}C]RPII plus cold RPI ($P < 0.05$).

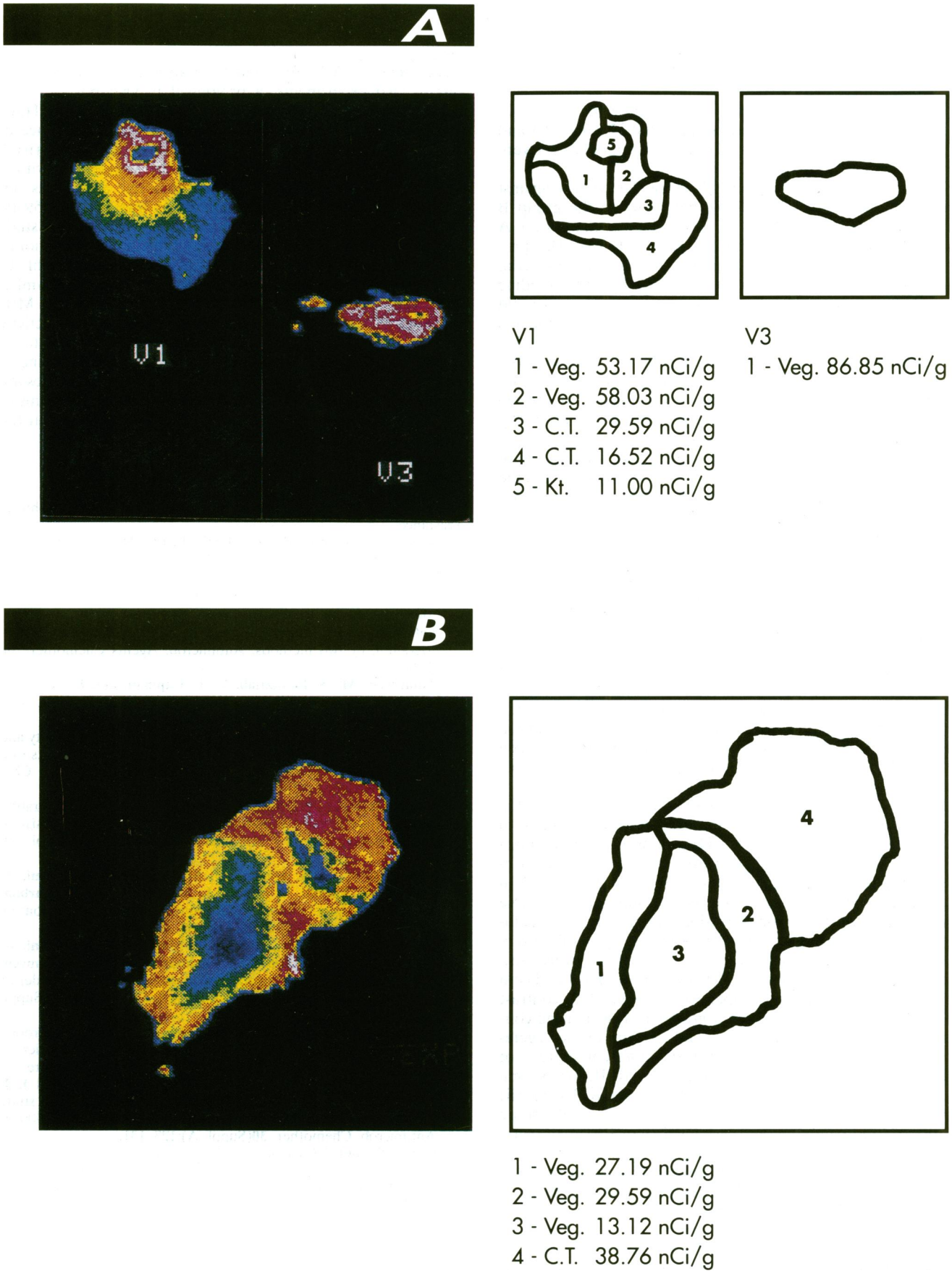

FIG. 2. Quantitative autoradiograph of vegetation and adjacent cardiac tissue taken from rabbits treated with [^{14}C]RPI plus cold RPII (A) or with [^{14}C]RPII plus cold RPI (B), 30 min after the end of intravenous infusion. The diagrams show the radioactivity (nanocuries per gram) of labelled antibiotics for each zone indicated on the autoradiographs. Veg., vegetation; C.T., cardiac tissue; Kt., catheter.

DISCUSSION

We demonstrated in this study that a synergistic antimicrobial activity could be observed between RP 57669 (RPI) and RP 54476 (RPII) in vitro and in vivo, in a model of severe staphylococcal infection.

The in vitro bacteriostatic and bactericidal synergy against staphylococci between RP 57669, belonging to the group B streptogramins, and RP 54476, belonging to the group A streptogramins, has been reported previously (3). The proposed mechanism is that the binding of RPII to the bacterial ribosome leads to a conformational modification at the binding site of streptogramins (2). This results in a 5- to 10-fold decrease in the dissociation constant between RPI and the 50S subunit of bacterial ribosome. As a consequence, a very stable RP 57669-ribosome-RP 54476 ternary 1:1:1 stoichiometric complex is formed (2). This mechanism of action could explain the synergistic activity against bacterial pathogens and also the prolonged postantibiotic effect observed in vitro for RP 59500 against staphylococci (15).

In experimental staphylococcal endocarditis, the absence of activity of RPI and RPII alone used in a 30-mg/kg dose and the reduction of almost 3 $\log_{10}$ CFU/g of vegetation obtained with RPI (9 mg/kg) when combined with RPII (21 mg/kg) as RP 59500 (30 mg/kg) probably reflected an in vivo synergism (9). This in vivo synergism was observed despite a different diffusion behavior of each labelled component of RP 59500 into the vegetation of experimental endocarditis. This might be explained by the fact that the synergy between RPI and RPII has been obtained in vitro and in different experimental murine models of infections due to *S. aureus* with a wide range of ratios of RPI and RPII, from 1:9 to 9:1 (3). Therefore, even if RPI diffused two to four times more than RPII into the core of the vegetation, due to the 30:70 ratio of RPI and RPII included in RP 59500 injected intravenously, the actual ratio between RPI and RPII in the core of the vegetation would be between 0.8 and 1.7, a ratio which is consistent with an achievable synergy in situ.

Radiolabelled RPI, the macrolide-like component of the combination, demonstrated a diffusion pattern comparable to that previously described with [^{3}H]spiramycin, another macrolide (6). The two macrolides were homogeneously distributed throughout the vegetation and more concentrated in vegetations than in cardiac tissue. Furthermore, considerable differences in antibiotic concentration among different vegetations in a single animal has been reported for [^{3}H]spiramycin (6). In the present study, important variations were also observed for [^{14}C]RPI and [^{14}C]RPII when cold RPII and cold RPII were coadministered, respectively (Table 3). In contrast to [^{14}C]RPI, [^{14}C]RPII showed a gradient of decreasing concentrations between the periphery and the core of the vegetation. Two factors may account for the different pattern of diffusion of RPII compared with that of RPII: (i) plasma protein binding is 60% for RPII and 30% for RPI (11); (ii) RPII and its metabolite, RP12536, have, unlike RPI, some degree of lipid solubility, and less RPII than RPI may penetrate into fibrin-rich foci such as cardiac vegetations.

RP 59500 was as active as vancomycin in the experimental model of endocarditis in rabbits, despite a dosing regimen which resulted in a prolonged period of time with antibiotic levels in serum below the MIC. This might be explained by different parameters. (i) A prolonged postantibiotic effect, up to 5 h, has been observed in vitro against methicillin-resistant strains of *S. aureus* exposed for 80 min to RP 59500 at four times the MIC (15); since the peak serum levels of RP 59500 were six times the MIC for the study strain and serum levels were above the MIC for almost 4 h after a single injection in our in vivo experiments, a prolonged in vivo postantibiotic effect probably allowed an intermittent dosing regimen. However, further studies are needed to evaluate the influence of the dosing regimen on the in vivo activity of RP 59500. (ii) A good penetration of RP 59500 into the vegetation, with a vegetation/blood ratio of 4 by microbiologic assay, was obtained. (iii) Finally, an important point was that RP 59500 demonstrated in vitro bactericidal activity against the study strain. This strain was representative of the in vitro behavior of RP 59500 against 10 clinical strains of *S. aureus* susceptible to erythromycin, with a killing rate of 3 $\log_{10}$ to 4 $\log_{10}$ CFU/ml at 24 h with concentrations equal to two to four times the MIC (data not shown). Although RP 59500 retains in vitro activity against most *S. aureus* organisms, whatever the resistance phenotypes to other antibiotics and to the macrolide-lincosamide-streptogramin family (12, 17), further in vivo studies are needed to evaluate the influence of the various mechanisms of resistance on the in vivo activity of RP 59500, in particular the constitutive resistance to erythromycin.

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