

Amendment history:

- [Correction](#) (April 1982)

Vancomycin prophylaxis of experimental *Streptococcus sanguis*. Inhibition of bacterial adherence rather than bacterial killing.

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J Clin Invest. 1981;[68\(4\)](#):1113-1116. <https://doi.org/10.1172/JCI110337>.

Research Article

Using a strain of *Streptococcus sanguis* tolerant to vancomycin to infect aortic vegetations in rats, we found that prophylactic intravenous vancomycin given 30 min before bacterial challenge decreased the incidence of endocarditis from 88 to 8% (P less than 10^{-5}). Because peak vancomycin serum levels were below the minimal bactericidal concentration, mechanisms of protection other than bacterial killing were investigated. *S. sanguis* were incubated with inhibitory concentration of vancomycin (50 microgram/ml) for 10 h and washed. 85% of rats (73/86) inoculated with control bacteria developed endocarditis, whereas only 42% (33/78) of those inoculated with vancomycin-exposed bacteria did so (P less than 10^{-5}). When rats were killed 30 min after bacterial challenge, *S. sanguis* were detected by culture of the vegetations in 44% of rats injected with control bacteria, but in only 13% of those challenged with vancomycin-exposed bacteria (P less than 0.03). Enhanced clearance of vancomycin-exposed streptococci was not responsible for this protection because blood cultures showed no difference in the level and duration of bacteremia after injection of control or vancomycin-exposed *S. sanguis*. Moreover, this protection was not abolished in neutropenic rats injected with vancomycin-exposed bacteria, despite more prolonged [...]

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Vancomycin Prophylaxis of Experimental *Streptococcus Sanguis*

INHIBITION OF BACTERIAL ADHERENCE RATHER THAN BACTERIAL KILLING

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ABSTRACT Using a strain of *Streptococcus sanguis* tolerant to vancomycin to infect aortic vegetations in rats, we found that prophylactic intravenous vancomycin given 30 min before bacterial challenge decreased the incidence of endocarditis from 88 to 8% ($P < 10^{-5}$). Because peak vancomycin serum levels were below the minimal bactericidal concentration, mechanisms of protection other than bacterial killing were investigated. *S. sanguis* were incubated with inhibitory concentration of vancomycin (50 $\mu\text{g/ml}$) for 10 h and washed. 85% of rats (73/86) inoculated with control bacteria developed endocarditis, whereas only 42% (33/78) of those inoculated with vancomycin-exposed bacteria did so ($P < 10^{-5}$). When rats were killed 30 min after bacterial challenge, *S. sanguis* were detected by culture of the vegetations in 44% of rats injected with control bacteria, but in only 13% of those challenged with vancomycin-exposed bacteria ($P < 0.03$). Enhanced clearance of vancomycin-exposed streptococci was not responsible for this protection because blood cultures showed no difference in the level and duration of bacteremia after injection of control or vancomycin-exposed *S. sanguis*. Moreover, this protection was not abolished in neutropenic rats injected with vancomycin-exposed bacteria, despite more prolonged bacteremia. These results suggest that vancomycin exerted its protection by lowering adherence of tolerant *S. sanguis* to vegetations rather than through bactericidal activity or enhanced clearance of bacteria by phagocytic cells. In the choice of antibiotics for prophylaxis of endocarditis, reduction of bacterial adhesion may be a criterion as important as bacterial killing.

Received for publication 1 July 1981 and in revised form 31 July 1981.

Portions of this work have been presented in abstract form at the 11th International Congress of Chemotherapy, Florence, Italy, July 1981.

INTRODUCTION

Recommendations for antibiotic prophylaxis of bacterial endocarditis in humans derive from studies in rabbits (1). These studies suggested that high and prolonged doses of a bactericidal antibiotic are necessary to achieve efficient prophylaxis (2). Furthermore, combinations of antibiotics were superior to single drug prophylaxis (2).

Vancomycin has proved to be one of the most successful antibiotics in the prevention of *Streptococcus viridans* endocarditis in rabbits and rats (2, 3). A report suggesting the lack of bacterial killing by cell wall inhibitors on some strains of *S. viridans* (4) prompted us to further investigate the mode of action of vancomycin in the prophylaxis of endocarditis in rats. By using a *S. sanguis* that was inhibited but not killed by vancomycin, it appeared that vancomycin did not exert its protective effect by direct killing, which until now was considered as a prerequisite for prophylaxis, but possibly by inhibiting bacterial adherence to vegetations.

METHODS

Microorganism. A previously described strain of *S. sanguis* was used (5). Minimal inhibitory and bactericidal concentration of vancomycin (Eli Lilly & Co., Indianapolis, Ind.), were determined by broth dilution tests (6). The minimal bactericidal concentration was defined as the lowest concentration of antibiotic that produced 99.9% killing. Killing curves with 50 $\mu\text{g/ml}$ of vancomycin were performed in trypticase soya broth (Difco Laboratories, Detroit, Mich.) using an inoculum of 10^6 colony-forming units (CFU)/ml of an overnight culture of *S. sanguis*. A concentration of 50 $\mu\text{g/ml}$ of vancomycin was chosen because it was similar to serum levels achieved 1 h after the intravenous injection of the prophylactic dose of 30 mg/kg.

¹Abbreviations used in this paper: CFU, colony-forming units; SS, control *Streptococcus sanguis*; VSS, vancomycin-exposed *S. sanguis*.

Production of endocarditis and evaluation of infection. Sterile vegetations were produced in female Wistar rats (180–200 g) by a modification of a described method (3, 5). Briefly, a polyethylene catheter (PP 10, Portex LTD, England) was inserted across the aortic valve through the right carotid artery and secured with a silk ligature. 24 h after catheterisation rats were injected in the tail vein with 0.5 ml of saline containing 10^6 CFU of *S. sanguis*. Rats were then killed at intervals and 1 ml of blood was drawn from the inferior vena cava and plated on blood agar. Aortic vegetations were excised, weighed, homogenized in 1 ml of saline, and serially diluted and plated. Plates were counted after 48 h of incubation at 37°C. This method permitted the detection of 10^2 CFU/g of vegetation.

Prophylaxis of *S. sanguis* endocarditis by vancomycin. 30 min before bacterial challenge with 10^6 CFU of *S. sanguis* from an overnight culture, rats were injected intravenously with either 30 mg/kg of vancomycin or saline. They were killed 24 or 72 h later as described above.

Vancomycin serum levels and serum bactericidal activity. Serum levels of vancomycin 30 min after injection of 30 mg/kg i.v. were determined by the agar diffusion technique (7) and serum bactericidal activity by standard methods (8).

Production of endocarditis with *S. sanguis* exposed to inhibitory concentration of vancomycin. In some experiments, *S. sanguis* was exposed to inhibitory concentration of vancomycin before injection to rats as follows: 10^7 CFU/ml of *S. sanguis* of an overnight culture were incubated at 37°C in trypticase soya broth with and without 50 µg/ml of vancomycin. After 10 h, the bacteria were washed on a Millipore filter (0.45 µm, Millipore Corp., Bedford, Mass.) with saline and resuspended in trypticase soya broth to obtain for both control *S. sanguis* (SS) and vancomycin-exposed *S. sanguis* (VSS), an inoculum of 10^6 CFU/0.5 ml. In each experiment the inocula were checked by serial plating; in addition, to test the ability of SS and VSS to grow after washing, aliquots were reincubated and plated after 2, 6, and 24 h of incubation.

Production of granulocytopenia. In some experiments neutropenia was produced by the injection of 100 mg/kg i.p. of cyclophosphamide (Endoxan-Asta, Asta Werke AG, Bielefeld, Germany) 3 d before aortic catheterisation.

Statistical evaluation. The Chi-square test with Yates' correction and the unpaired Student's *t* test were used for statistical comparisons.

RESULTS

Susceptibility of *S. sanguis* to vancomycin. Minimal inhibitory and bactericidal concentration of the *S. sanguis* for vancomycin were 0.5 and 128 µg/ml, respectively. Survival of *S. sanguis* in 50 µg/ml of vancomycin showed no significant decrease in CFU (<50% killing) during the first 10 h in all of several experiments; 90–99% killing occurred after 24 h. Therefore, this *S. sanguis* was tolerant to the bactericidal action of vancomycin. Moreover, when washed after 10 h of exposure to vancomycin, *S. sanguis* so treated showed growth at 2, 6, and 24 h that was similar to that of unexposed *S. sanguis*.

Vancomycin levels and serum bactericidal activity. Vancomycin serum levels (\pm SD) in 5 rats 30 min and 1 h after injection of 30 mg/kg i.v. were respectively 77 ± 21 and 47 ± 8 µg/ml. There was no detectable serum bactericidal activity 30 min after injection of van-

comycin, that is at the time of injection of bacteria in the prophylaxis experiments.

Prophylaxis of *S. sanguis* endocarditis by intravenously administered vancomycin. The incidence of endocarditis was reduced from 88% (46/52 rats) in controls to 8% (2/26) in rats killed at 24 h and 6% (1/18) in rats killed at 72 h ($P < 10^{-5}$).

Ability of vancomycin-exposed *S. sanguis* to produce endocarditis. As shown on Fig. 1, *S. sanguis* exposed to inhibitory concentrations of vancomycin (VSS) produced infection of vegetations less frequently than SS. In animals killed at 24 h, the incidence of endocarditis was reduced from 85% (73/86) to 42% (33/78), ($P < 10^{-5}$), with no significant difference in the mean bacterial density of infected vegetations between the two groups. In rats killed 30 min after bacterial challenge to investigate early infection of vegetations, detectable bacteria were found in only 13% of rats (3/24) after VSS challenge, compared with 44% (12/27) after SS challenge ($P < 0.03$), (Fig. 1).

Clearance of vancomycin-exposed *S. sanguis* from the blood. To investigate if preincubation of *S. sanguis* with vancomycin might enhance their clearance from the blood and could account for protection, blood cultures were performed in catheterised rats

Log ₁₀ MBD	=	3.15	3.13	3.33	5.93	5.33	5.19
\pm SD		0.40	0.62	0.45	1.43	1.31	1.01

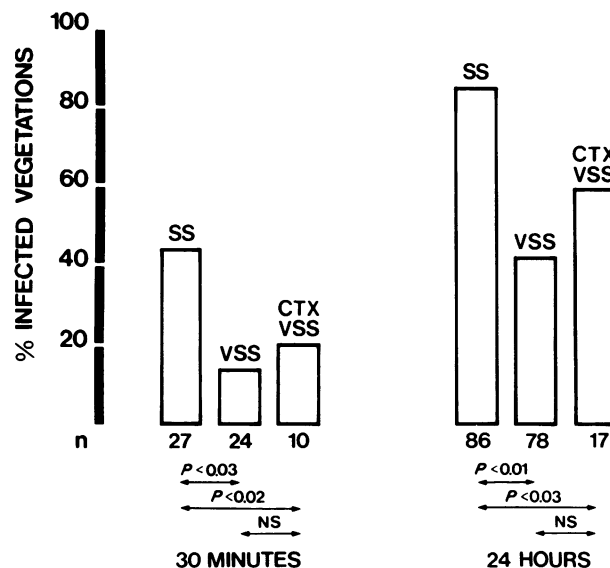


FIGURE 1 Incidence of infection of vegetations 30 min and 24 h after intravenous injection of vancomycin-exposed *S. sanguis* (VSS) or control *S. sanguis* (SS). The CTX columns represent groups of rats treated with cyclophosphamide before bacterial challenge with VSS. *n* represents the total number of rats in each group. MBD is the mean bacterial density (\pm SD) recovered from infected vegetations in each group of rats. The difference in the incidence of infection between groups of rats was evaluated by the Chi-square test with Yates' correction. NS = not significant.

early after challenge. As shown in Fig. 2 the incidence and severity of bacteremia at 10 and 30 min was similar after injection of VSS or SS. Thus, next to showing similar blood clearance for VSS and SS, these experiments confirmed that, when injected, VSS were as viable as SS.

Effect of neutropenia on clearance of bacteria from the blood, on colonization of vegetations, and on the development of endocarditis. To determine whether VSS remained less infective in rats with more severe and sustained bacteremia, control and neutropenic rats were injected with VSS. The rats treated with cyclophosphamide had a mean leukocyte count of $400/\text{mm}^3$, with 0–2% granulocytes at the time of bacterial challenge, and agranulocytosis persisted for >24 h. As shown on Fig. 2, this resulted in a significant prolongation of bacteremia after challenge with VSS, as compared with bacteremia observed in nonneutropenic rats. Despite this prolongation, no difference in the incidence of endocarditis was observed between control and neutropenic rats injected with VSS, and both groups were significantly different from control rats injected with SS (Fig. 1). Therefore, the protective effect of vancomycin exposure persisted in spite of cyclophosphamide treatment.

DISCUSSION

Current recommendations for antibiotic prophylaxis of bacterial endocarditis are based on studies in rabbits and aim at achieving high and prolonged serum bactericidal activity (1). Recently, strains of *S. sanguis*, a major cause of bacterial endocarditis in man, have been shown to be tolerant to the bactericidal action of penicillin and other cell wall inhibitors (4). In this study, we used a *S. sanguis* strain that was tolerant to

the bactericidal effect of vancomycin. The serum concentration of vancomycin at the time of bacterial challenge was not bactericidal in vitro, and serum bactericidal activity was undetectable. Nevertheless, vancomycin was an extremely efficient prophylaxis. Moreover, protection was observed when *S. sanguis* was preincubated with nonbactericidal concentration of vancomycin and washed before injection.

These observations suggest that mechanisms other than killing were responsible for the protective action of vancomycin. It has been shown that inhibitory or subinhibitory concentrations of antibiotics may promote phagocytosis of bacteria (9). If incubation with vancomycin had acted through this mechanism, one would have expected a more rapid clearance from the blood of vancomycin-exposed *S. sanguis* than of control bacteria. This hypothesis was disproved by the observation that incubation of *S. sanguis* with vancomycin did not change the severity and duration of bacteremia. Furthermore, protection after exposure to vancomycin persisted in the absence of neutrophils and despite more sustained bacteremia, and could therefore not be attributed to increased phagocytosis of vancomycin-exposed bacteria at the surface of the vegetations or elsewhere.

An alternative hypothesis is that vancomycin exerted its prophylactic effect by preventing the bacteria from adhering to vegetations. 30 min after bacterial challenge, bacteria were detected less often on vegetations when *S. sanguis* had been preexposed to vancomycin. Because their viability was apparently identical to unexposed bacteria as judged by similar incidence and level of bacteremia in vivo and by similar growth curves in vitro, it suggests that less vancomycin-exposed *S. sanguis* had adhered to the vegetations, with the final result of a decreased incidence of endocarditis. Adherence has been shown to play an important part in the pathogenesis of bacterial endocarditis (10). Furthermore, there is a body of evidence showing that antibiotics that inhibit cell wall synthesis reduce bacterial adherence. Experiments with *S. sanguis* have shown that incubation with nonbactericidal concentrations of penicillin (11) and vancomycin (12) decreases the adherence of bacteria to platelet-fibrin clots in vitro. Other studies have demonstrated that incubation of *S. sanguis* (4) or *S. pyogenes* (13) with inhibitors of cell wall synthesis induces the release of lipoteichoic acid, a cell wall constituent. Lipoteichoic acid has been shown to mediate adherence of group A Streptococci to epithelial cells, and depletion of lipoteichoic acid resulted in loss of bacterial adherence (13). The results of our experiments are compatible with, and extend these in vitro data, and suggest that bacteriostatic levels of vancomycin may prevent endocarditis by decreasing the adherence of *S. sanguis* to vegetations in vivo.

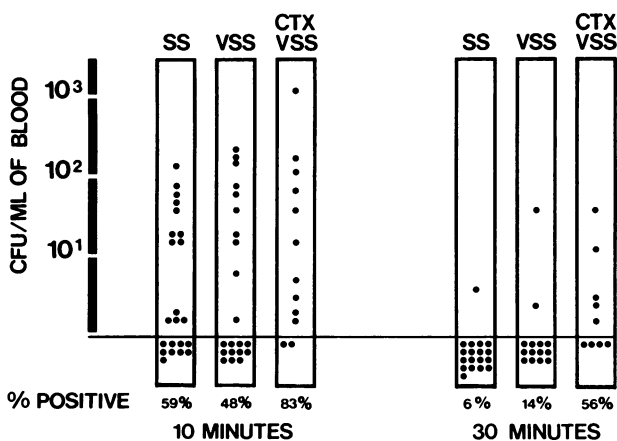


FIGURE 2 Incidence and severity of bacteremia 10 and 30 min after intravenous injection of vancomycin-exposed *S. sanguis* (VSS) or control *S. sanguis* (SS). CTX columns represent groups of rats treated with cyclophosphamide before bacterial challenge. Each point represents one rat.

In previous studies bacteriostatic antibiotics such as tetracycline failed to provide adequate prophylaxis in rabbits, and it was therefore concluded that bactericidal antibiotics were required to achieve protection (14). The discrepancy between these previous results and those obtained with nonbactericidal concentration of vancomycin can be attributed to the difference in the modes of action of the antibiotics rather than to the difference in their bactericidal activities; unlike vancomycin, tetracyclines are metabolic inhibitors with no significant effect on cell wall synthesis.

We conclude that the ability to reduce bacterial adherence may be an important criterion when choosing an antibiotic for the prophylaxis of endocarditis.

ACKNOWLEDGMENTS

We thank Professor L. R. Freedman for continued interest in this work, and Dr. J. A. McCutchan and Dr. P. Casey for critical review of the manuscript.

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