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Research Article

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In vitro experiments were performed using platelet-fibrin surfaces to test the possibility that penicillin-induced loss of lipoteichoic acid was responsible for decreased streptococcal adherence. Pretreatment of *S. sanguis* cultures with inhibitory concentrations of penicillin or with antiserum against lipoteichoic acid and precoating of the platelet-fibrin surfaces with lipoteichoic acid, […]

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ABSTRACT The effect of penicillin treatment of *Streptococcus sanguis* in vitro, on subsequent bacterial density in the bloodstream and on cardiac valves in the rabbit model of endocarditis was studied. As experimental tools for this study, isogenic pairs of *S. sanguis* differing in resistance to streptomycin or rifampin were prepared by genetic transformation. Rabbits with traumatized heart valves received an intravenous inoculation of penicillin treated (1 μg/ml) and untreated *S. sanguis*, each marked by resistance to either streptomycin or rifampin. The number of penicillin-treated and untreated bacteria attached to the valvular surfaces was determined by differential counting on streptomycin or rifampin containing media. Penicillin pretreatment reduced cardiac valve colonization 5 min after inoculation ("adherence ratio" $\times 10^8$ was 4.11 for the control and 3.66 for the penicillin-treated bacteria, $P < 0.001$). The results were not due to differences in serum killing or bacterial densities in the bloodstream. There was no difference in valvular bacterial densities 24 h after bacterial inoculation (adherence ratio $\times 10^8$, 7.26 untreated vs. 6.34 penicillin-pretreated, $P > 0.10$).

In vitro experiments were performed using platelet-fibrin surfaces to test the possibility that penicillin-induced loss of lipoteichoic acid was responsible for decreased streptococcal adherence. Pretreatment of *S. sanguis* cultures with inhibitory concentrations of penicillin or with antiserum against lipoteichoic acid and precoating of the platelet-fibrin surfaces with lipoteichoic acid, all caused reduction in bacterial adherence. The findings are interpreted as support for the role of lipoteichoic acid as an adhesin in *S. sanguis* interactions with particular host tissue surfaces.

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INTRODUCTION

Adherence of bacteria to a cardiac valvular surface is the initial event in the development of infective endocarditis. In experimental models of endocarditis bacteria selectively adhere to traumatized valvular surfaces covered with a platelet-fibrin matrix (1, 2). Gram positive bacteria adhere more readily to these surfaces than gram negative bacteria and, perhaps as a consequence, are more frequent pathogens in cases of endocarditis (3, 4).

This study is an investigation of the effect that inhibitory concentrations of penicillin have on bacterial adherence. Inhibitory or subinhibitory antibiotic concentrations have been shown to reduce bacterial adherence to host cell surfaces in vitro (5, 6). Clinically, a penicillin-mediated effect on bacterial adherence to cardiac valvular surfaces, independent of bacterial killing, could play an important role in the prevention of endocarditis.

Among the in vitro effects caused by penicillin, the loss of lipoteichoic acid (LTA)[1] into the medium may be an important factor in decreasing gram positive bacterial adherence (5). Beachey has demonstrated that LTA acts as a specific adhesin for group A streptococci, facilitating bacterial binding to a number of surfaces including epithelial cells, platelets, and erythrocytes (RBC) (7–9). Antibiotic-induced decrease in the adherence of *Streptococcus sanguis* and some other gram positive bacteria have already been observed (6) and therefore raise the possibility that LTA may also play an important role in mediating streptococcal attachment to valvular surfaces.

[1] *Abbreviations used in this paper*: CFU, colony-forming units; LTA, lipoteichoic acid; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; PFS, platelet-fibrin surface; RBC, erythrocytes; Rif, rifampin resistant; St, streptomycin resistant; THB, Todd Hewitt broth.

The bacterial species investigated in this study, *S. sanguis*, is one of the most common isolates in patients with streptococcal endocarditis (10). The strain is a naturally occurring "tolerant" bacterium, resistant to killing by penicillin in vitro even when exposed to high concentrations (11–13). While resistant to penicillin induced lysis, other effects of penicillin are still demonstrable: morphologic changes in cell structure, reduction in the rate of cell wall synthesis and most notably increased release of LTA into the medium (11–13). The tolerant strain was selected in order to study the effects of inhibitory concentrations of penicillin, independent of bacterial killing. The purpose of this study was to investigate the effects that inhibitory concentrations of penicillin have on streptococcal adherence and the extent to which loss of LTA from the cell accounts for these effects.

METHODS

Bacterial strain and growth. The *S. sanguis* (strain Wicky) and the streptomycin-resistant (St) and rifampin-resistant (Rif) transformant derivatives were used in all experiments. The bacteria were grown at 37°C in test tube cultures of Todd Hewitt broth (THB) (BBL Microbiology Systems, Cockeysville, MD). Bacteria grew in such cultures with doubling times of ~40 min.

Bacterial transformation. The Rif or St markers were introduced into the common genetic background of Wicky cells made "competent" to take up DNA (14, 15). DNA isolated from spontaneous antibiotic-resistant mutants was isolated (14, 15) and used to transform the competent bacteria. The resistant transformants were selected on Todd Hewitt agar plates containing either streptomycin sulphate (100 μg/ml) or rifampin (2 μg/ml). The minimal inhibitory concentration/minimal bactericidal concentration (MIC/MBC) results to penicillin G for the antibiotic-resistant strains were comparable to the original Wicky strain.

Antibiotic susceptibility testing. Broth dilution susceptibility tests were performed in duplicate using THB or pooled rabbit serum (16). The minimal bactericidal concentration (MBC) was determined by subculturing 0.1 ml from each tube onto sheep blood agar plates and incubating the plates for 48 h at 37°C.

In vitro penicillin treatment. Overnight cultures of *S. sanguis* were diluted 1/100 into prewarmed THB with or without pooled fresh rabbit serum. Penicillin was added to exponentially growing cultures at a cell concentration of about 10^7 colony-forming units (CFU)/ml. To test the effect of penicillin on bacterial viability, aliquots were removed for colony counting over 24 h and were treated with *Bacillus cereus* beta lactamase to minimize carryover of penicillin (17). Serial 10-fold dilutions were made and plated in duplicate on Todd Hewitt agar.

Rabbit model of endocarditis. Aortic valvular endocarditis in male New Zealand rabbits (1.8–2.3 kg) was produced using a modification of the method described by Perlman and Freedman (1, 18). A polyethylene catheter was passed across the aortic valve, tied in place, and the wound closed. The studies were performed 48 h after surgery.

Determination of in vivo adherence. Overnight cultures of the two antibiotic-resistant strains were diluted 1:100 into fresh, prewarmed THB and incubated at 37°C. One of the pairs of 10-ml cultures of the St and Rif strains received penicillin (1 μg/ml) when the cell concentration had reached ~10^7 CFU/ml. After 1-h optical densities of the control and penicillin-treated cultures were adjusted to equal density, centrifuged, washed twice in THB, and resuspended in 0.9% NaCl. Equal volumes of the two bacterial suspensions were mixed and a 1-ml aliquot of the mixed suspension was intravenously injected into each rabbit. To determine the initial cell concentration aliquots of the mixed suspensions were removed for colony counting by plating on Todd Hewitt agar containing either streptomycin (100 μg/ml) or rifampin (2 μg/ml). Rabbits were killed 5 min, 3 and 24 h after inoculation. All cardiac valvular tissue plus valvular vegetations were aseptically excised, homogenized in a tissue grinder (3431-E04 AA, Arthur H. Thomas Co., Philadelphia, PA) and serially diluted in normal saline. The dilutions were plated in duplicate using Todd Hewitt agar containing either no antibiotic, streptomycin (100 μg/ml), or rifampin (2 μg/ml) to allow for the identification of the bacterial populations derived from the penicillin-pretreated and untreated groups. Penicillin pretreatment of the two labeled strains was reversed for each experiment. The results were expressed as an "adherence" ratio, defined as the number of bacteria adherent to the valvular tissue expressed in $\log_{10}$ CFU/milliliter divided by the original inoculum multiplied by $\log_{10} 8$.

Effect of penicillin on bacterial density in the bloodstream. During the in vivo study venous blood samples were obtained to determine if penicillin pretreatment affected the bacterial density in the bloodstream. Samples (1.5 ml) were taken at 1, 5, 10, 30, 60 min, 3 and 24 h. The specimens were divided into 0.5-ml aliquots, diluted, and plated onto Todd Hewitt agar containing either streptomycin, rifampin, or no antibiotic.

Preparation of the platelet-fibrin surface (PFS). The PFS was prepared using the technique described by Scheld et al. (19).

Adherence assay. An overnight culture of the *S. sanguis* strain was diluted 1:100 into fresh prewarmed THB and incubated at 37°C for 2 h. In experiments measuring the effect of penicillin on bacterial adherence the bacterial suspension was divided into equal aliquots after determining the optical density (model 620A linear spectrophotometer, Coleman Instruments, Oak Brook, IL). Penicillin was added to one of the suspensions and the two test tubes, with 5 ml culture in each, were further incubated for 1 h at 37°C. The optical densities of the two suspensions were then equalized and a 10^5 dilution made in THB, resulting in a final bacterial concentration of 10^3 CFU/ml. 5-ml aliquots of these diluted suspensions were added to the PFS and were agitated at 120 rpm on a Junior Orbit Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) at 37°C. The bacterial suspension was then decanted and the PFS washed three times for 5 min with prewarmed THB (5 ml) at 37°C. Samples from the original suspension and the washes were taken for bacterial colony counting. The same bacterial concentrations and fluid volumes were used in all in vitro studies. The PFS was gently overlaid with Todd Hewitt agar containing penicillinase and incubated for 48 h at 37°C. Colonies adherent to the PFS were counted. All experiments included both control and penicillin-treated samples. The results were calculated as the number of colonies adherent to the clot divided by the original bacterial inoculum (expressed in CFU/milliliter) multiplied by 100 and were expressed as an adherence ratio. The reproducibility of these experiments showed greater variation when performed on separate days than on the same day. These differences were in part due to the use of platelets

from different volunteers, as well as variation in the absolute number of bacteria used in the initial inoculum.

Using the in vitro adherence assay described above a comparison was made of the three *S. sanguis* strains (Wicky, Wicky Rif, and Wicky St) to determine if there was any demonstrable difference in adherence to the PFS. The surface was exposed to 1 of the 3 strains for 15 min; washed and overlaid with agar. A comparison of 11 paired samples revealed no differences among the three groups ($P > 0.05$).

Standardization of adherence assay. Bacterial suspensions were vortexed to reduce bacterial aggregation and chaining. This technique was found to be equally effective to filtration of bacteria through an 8-μm Millipore filter (Whatman, Inc., Clifton, NJ) or passage of bacteria through a sterile 25-gauge syringe. A comparison of viability between the untreated and penicillin-pretreated groups was made after completing the adherence assay. A "wash ratio" calculated as the number of bacteria adherent to the clot plus the total number of bacteria recovered from the three washes divided by the initial bacterial inoculum was determined. This ratio ideally should be 1. The mean ratios for 16 pairs of untreated-penicillin and treated groups were 0.94±0.2 and 0.85±0.2, respectively ($P > 0.10$).

Elution studies. The ability of penicillin to influence the elution of streptococci from the PFS was studied. A bacterial suspension, prepared as described above, was added to the PFS and agitated for 15 min at 37°C. The suspension was then decanted and the PFS overlaid with 5-ml aliquots of THB or 0.05 M Tris maleate buffer, pH 7 (Sigma Chemical Co., St. Louis, MO), each with or without penicillin (1 μg/ml). The plates were shaken on a Junior Orbit Shaker at 37°C and the supernatant changed every 15 min. After a total of 1 h incubation (four changes of supernatant) the PFS was washed twice for 5 min, the first wash still containing penicillin (if the clot had been penicillin treated) then overlaid with Todd Hewitt agar containing penicillinase, and incubated for 48 h. "Wash ratios" were again calculated. Controls for this study included plates that were immediately overlaid with agar and not washed with either THB or buffer after exposure to bacteria.

Preparation of LTA. LTA was prepared from the Wicky strain by phenol extraction (20). The preparation was fractionated on a Bio-gel A-5m column (Bio-Rad Laboratories, Richmond, CA; 1.5 × 50 cm). Aliquots were assayed by the phosphate determination technique of Ames and Dubin (21).

The RBC sensitizing activity and the antigenic activity of the LTA were assayed using the method of Ofek et al. (7).

Preparation of LTA antisera used for adherence studies. LTA used for the production of antisera was prepared using the chloroform-methanol extraction method of Wicken et al. (20). Rabbits were immunized using a modification of the method of Burger (20, 22).

The presence of antisera with specific anti-LTA activity was detected using three techniques; agar gel immunodiffusion, quantitative precipitin, and RBC sensitization. LTA and anti-LTA (kindly provided by Dr. Wicken, University of New South Wales, Australia and by H. Courtney, Veterans Administration Hospital, Memphis, TN) were used as controls in these studies. The agar gel diffusion test was performed using Agar Noble (Difco Laboratories, Detroit, MI) combined with sodium chloride (1/0.45, wt/wt) and poured onto microscopic slides. Wells were punched and 10-μl samples added to the wells. Undiluted antisera was placed in the central well, with serial twofold dilutions of LTA starting with an initial concentration of 16,000 μg/ml in the surrounding wells. The plates were incubated at 37°C overnight and read after 24 h.

The quantitative precipitin test was performed using the methods of Knox et al. (23) and McCarty and Lancefield (24). Folin-Ciocalteus' phenol reagent (1.2 ml) was used as described by Heidelberger and MacPherson (25) for the estimation of serum antibody.

The RBC sensitization assay, described above, was also used to demonstrate anti-LTA activity using a fixed quantity of LTA (100 μg/ml) and serial twofold dilutions of antisera.

Effect of LTA on adherence. The in vitro adherence assay (19) was used to determine the effect of purified LTA on the adherence of *S. sanguis* to the PFS. In these studies LTA (3 ml, 100 μg/ml) dissolved in 0.05 M Tris maleate buffer, pH 7, was added to the PFS, the plates incubated at 37°C for 0.5 h and then, prior to the addition of bacteria, the supernate removed. Tris buffer was used as a control.

The effects of antisera on bacterial adherence was studied. A 1/100 dilution of an overnight growth of *S. sanguis* was incubated at 37°C for 1 h and then back diluted 10^6 into buffer (0.05 M Tris-maleate, pH 7.0) containing various dilutions of the antisera. These suspensions were incubated for an additional 0.5 h before performance of the adherence assay. Pooled rabbit serum obtained from the animals before immunization was used as a control. Microscopic examinations of the culture treated with LTA or antisera under the conditions described demonstrated no observable clumping of the bacteria.

Statistical evaluation. Both in vitro and in vivo results were compared using the paired Student's *t* test. Results were expressed as the mean±SEM.

RESULTS

Antibiotic susceptibility. Broth dilution susceptibility tests to penicillin G for the *S. sanguis* used in these studies revealed an MIC/MBC of 0.05/6.2 μg/ml in THB and 0.1/12.5 μg/ml in pooled rabbit serum. There was a 128-fold difference between the MIC and MBC.

Effect of penicillin treatment on viability and LTA release in vitro. Fig. 1 shows the effect of penicillin treatment on the viable titer of the cultures that were subsequently used for the in vivo studies. The results with the transformant cells were similar to those of the parent cells (Wicky strain); there was minimal bacterial killing over the first 4 h of exposure to the antibiotic. Even after 24 h of penicillin treatment there was less than 1 log killing at the 1-μg/ml concentration. Addition of fresh or heated rabbit serum at 48% or exposure of the cells to penicillin in 95% serum did not alter these results.

Effect of in vitro penicillin pretreatment on the adherence of S. sanguis to rabbit heart valves. Blood samples taken at frequent intervals after bacterial inoculation demonstrated no significant difference in the concentration of the penicillin-treated and untreated cells (Fig. 2).

Rabbits were killed 5 min, 3 and 24 h after the inoculation. Heart valves were removed and the titer of the penicillin-treated and control streptococci were determined by plating on selective agar. Table I summarizes data from a number of experiments. Although there was a substantial variation from experiment to experiment, the antibiotic pretreatment caused a de-

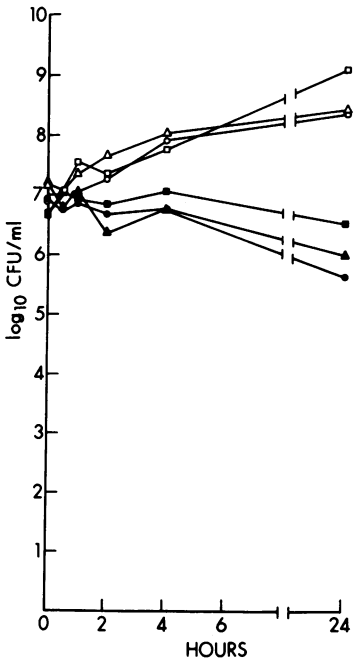

FIGURE 1 Time-kill study using the tolerant *S. sanguis* (Wicky) strain performed in varying concentrations of rabbit serum. The effect of penicillin (1 μg/ml; closed symbol) is compared with control (open symbol). Three concentrations of rabbit serum were used: 0 (○), 48 (△), and 95 (□) %.

crease in the number of adherent bacteria in the earliest (5 min) samples ($P < 0.001$) and in the 3-h samples ($P < 0.001$). There was no significant difference in the 24-h samples ($P > 0.10$).

During the in vivo experiments the strain exposed to penicillin was reversed for each experiment. The results were similar, regardless of which strain was pretreated with penicillin.

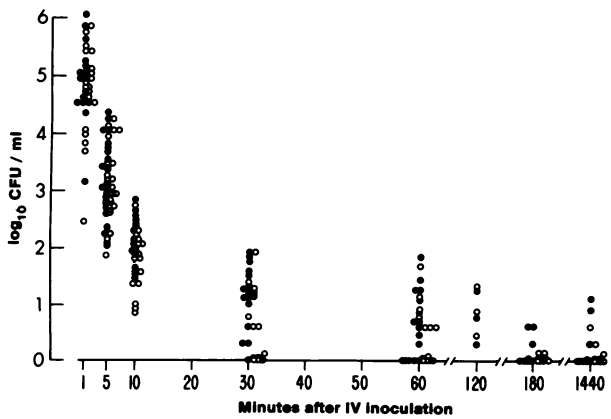

FIGURE 2 The density of bacteremia following the intravenous inoculation of the tolerant *S. sanguis* strain. One-half of this suspension was pretreated in vitro with penicillin 1 μg/ml (○). The remainder were unexposed to penicillin (●).

Effect of penicillin pretreatment on in vitro adherence to PFS. Preincubation of the tolerant *S. sanguis* with inhibitory concentrations of penicillin (1 μg/ml) resulted in decreased bacterial adherence to the PFS. This decrease could be demonstrated if the bacteria were allowed to interact with the PFS for at least 15 ($P < 0.05$) or 30 min ($P < 0.01$). No decrease was observable after shorter time periods (e.g., 1 or 5 min, $P > 0.05$) (Table II). The adherence ratio increased for both control and treated bacteria groups as they were exposed to the PFS for a longer period of time.

Preincubation of *S. sanguis* with a range of concentrations of penicillin for 15 min resulted in significantly decreased adherence with concentrations of 1 and 10 μg/ml ($P < 0.05$) but not with 0.025 μg/ml, a concentration below the MIC of the organism (Table III).

Elution of bacteria from PFS. In these studies the ability of penicillin containing medium to elute attached *S. sanguis* from the surface was determined (Table IV). The results (Table IV) show that (*a*) there were significantly larger numbers of adherent colonies in the plates incubated with penicillin-free THB ($P < 0.01$) than in those that were incubated with penicillin containing THB. The wash ratios (see Methods) were 1.2 vs. 1.06 ($P < 0.01$) for the penicillin-free and penicillin-containing groups, respectively. Furthermore, (*b*), the number of colonies in the plates incubated with THB containing penicillin were the same as in the control plates that were not postincubated at all. There were about the same number of colonies on the control plate and on the plates postincubated with Tris buffer with or without the antibiotic. The wash ratios were 0.93 and 0.94, respectively.

Determination of LTA activity. The activity of the purified LTA was 8182 by the RBC sensitizing assay and 16 by the hemagglutination inhibition assay (expressed as the reciprocal of the highest dilution of LTA capable of either causing or inhibiting visible agglutination, respectively). The LTA preparation used to immunize the rabbits demonstrated titers of 256 and 2 for the RBC sensitization and hemagglutination inhibition assay, respectively.

Determination of anti-LTA activity. The anti-LTA activity of the rabbit sera was 1024 by RBC sensitization, 250 by immunodiffusion (expressed as the lowest dilution of LTA [in micrograms per milliliter] which resulted in the demonstration of a detectable precipitin line after 24 h) and 0.8 by quantitative precipitin (expressed as the maximal amount of precipitated protein divided by the amount of serum used [milligrams per milliliter]). The activity of the control sera was 0.0 and 0.4 for the three assays, respectively.

Effect of LTA on adherence. Preexposure of the PFS to LTA (100 μg/ml) for 15 min significantly re-

TABLE I

Mean Bacterial Densities on Cardiac Valvular Tissue after the Intravenous Inoculation of Mixtures of Penicillin Pretreated (1 μg/ml) and Control S. sanguis into Rabbits

Results 5 min after inoculation							
	Inoculum*			Bacterial density*		Adherence ratio‡	
Experiment	Control	Treated	Rabbit	Control	Treated	Control	Treated
1	7.08 (Rif)	6.9 (St)	1	4.19	2.83	5.11	3.93
			2	2.3	1.3	3.22	2.4
			3	3.34	2.41	4.26	3.51
			4	3.98	2.60	4.90	3.71
2	7.47 (St)	7.82 (Rif)	1	3.08	2.98	3.61	3.16
			2	3.18	3.27	3.71	3.45
			3	3.33	3.37	3.86	3.55
3	7.21 (St)	7.56 (Rif)	1	3.60	3.67	4.39	4.11
			2	3.28	3.42	4.07	3.86
4	7.51 (Rif)	7.26 (St)	1	3.51	2.42	4.0	3.16
			2	3.87	3.63	4.36	4.37
			3	3.48	3.32	3.97	4.06
			4	2.86	2.63	3.85	3.37
			5	3.65	3.0	4.14	3.74
5	7.2 (St)	7.75 (Rif)	1	2.40	2.67	3.2	2.92
			2	4.13	4.18	4.93	4.43
			3	3.73	3.82	4.53	4.07
			4	3.65	3.82	4.45	4.07

		Adherence ratio		
	Bacterial inoculum*	5 min§	3 h	24 h
Control	7.23±0.06	4.11±0.13	4.02±0.23	7.26±0.33
		(18)‖	(13)	(12)
Penicillin Treated	7.27±0.13	3.66±0.12	2.06±0.26	6.34±0.26
	$P > 0.10$	$P < 0.001$	$P < 0.001$	$P > 0.10$

* Mean bacterial inoculum and the concentration of adherent bacteria were expressed in $\log_{10}$ CFU/ml±SE.

‡ The adherence ratio represents the number of bacteria adherent to the valvular tissue expressed in $\log_{10}$ CFU/ml±SE divided by the original inoculum ($\log_{10}$ CFU/ml) multiplied by $\log_{10}8$.

§ The time after the bacterial injection when the rabbits were killed.

‖ Number of rabbits.

duced bacterial adherence ($P < 0.01$). An LTA concentration of 1,000 μg/ml did not significantly change the results. When the time interval of bacterial exposure to the PFS was reduced from 15 to 3 min the effect of LTA was still demonstrable (Table V). Exposure of the PFS to buffer containing LTA (100 μg/ml) resulted in a decrease in RBC sensitizing activity in the buffer from 1,024 to 2.

Effect of anti-LTA on adherence. Preincubation of *S. sanguis* with a 1/10 dilution of anti-LTA prior to exposure to the PFS significantly reduced adherence when compared with pooled rabbit serum ($P < 0.02$) (Table VI). Bacterial adherence was not reduced when the PFS was exposed to a 1/10 dilution of anti-LTA prior to the addition of bacteria. No differences in adherence were demonstrable when a 1/100 dilution of antisera was used.

When supernatant THB obtained from a culture of penicillin-treated *S. sanguis* was exposed to the PFS for 0.5 h before the addition of bacteria for 15 min the adherence ratio was reduced from 30.6±2.7 with control THB to 26.4±2.2 with the penicillin-treated THB (15 pairs, $0.10 > P > 0.05$). The RBC sensitizing titer in the treated supernatant was 64–128 prior to expo-

TABLE II
Effect of Pretreatment with Penicillin (1 μg/ml) on the Adherence of S. sanguis to a Platelet Fibrin Surface

Treatment group	Adherence ratio* 1 min‡	5 min	15 min	30 min
Untreated	9.4±1.7	19.2±3.2	19.0±1.1	36.9±3.8
	(16)§	(14)	(16)	(15)
Penicillin pretreated	8.4±1.4	15.2±2.8	13.3±0.9	29.2±3.3

* The adherence ratio represents the mean±SE of the number of colonies adherent to the clot divided by the initial bacterial inoculum (expressed in CFU per milliliter) multiplied by 100.
‡ The time indicates the number of minutes bacteria were in contact with the platelet-fibrin surface.
§ The number of parenthesis represents the number of paired samples assayed.

sure to the PFS and 1 after. The control THB titer was 0.

Since penicillin treatment causes loss of cellular LTA (11, 13) attempts were made to "coat" bacteria with isolated LTA after the antibiotic treatment. The adherence ratios of penicillin pretreated LTA coated *S. sanguis* returned to the level of the controls (Table VII). The adherence ratio for the penicillin pretreated bacteria remained significantly lower than either the control or the LTA treated group ($P < 0.05$).

DISCUSSION

Angrist and Oka (26) proposed that the initial lesion in bacterial endocarditis is a sterile vegetation or nonbacterial thrombus consisting of platelets and fibrin, which forms on damaged heart valves. Bacterial attachment to these surfaces is the first step in the pathogenesis of endocarditis. The present study investigates the effect that pretreatment of *S. sanguis* with penicillin has on this attachment utilizing traumatized rabbit heart valves in vivo and the PFS in vitro. Both surfaces have been demonstrated microscopically to structurally resemble the nonbacterial thrombus (2, 19).

Several factors that influence bacterial adherence to valvular vegetations have been studied. Gould et al. (3) found that gram positive cocci adhere more readily to heart valve surfaces than other bacteria. Both Scheld et al. (19) and Ramirez-Ronda (27) found that dextran-producing strains of streptococci adhere better than nonproducers. In this communication we present evidence suggesting that penicillin can interfere with bacterial adherence to valvular surfaces independent of bacterial killing.

Our in vivo studies demonstrated that pretreatment with penicillin decreased colonization of rabbit valvular vegetations after 5 min and 3 h but not after 24 h. The data was not explained by increased serum killing (Fig. 1) or by a lower bacterial density in the bloodstream (Fig. 2) of the penicillin pretreated strains. These results are consistent, therefore, with a decrease

TABLE III
*Effect of Pretreatment with Varying Concentrations of Penicillin on the Adherence of S. sanguis to a Platelet Fibrin Surface**

Penicillin pretreatment	Number of observations	Adherence ratio
μg/ml		
0	9	32.0±3.3
0.025	9	31.8±4.0
1	9	27.2±2.9
10	9	23.7±3.2

* Bacteria were exposed to the platelet-fibrin surface for 15 min.

TABLE IV
Effect of Penicillin (1 μg/ml) on the Elution of S. sanguis from the Platelet Fibrin Surface

Treatment group	Adherence ratio THB	Tris*
Untreated	36.9±3.7 (16)‡	49.7±4.3 (12)
Penicillin treated	23.9±2.5 (16)	46.5±1.4 (12)
Control§	23.5±2.3 (7)	50.7±3.7 (6)

* Buffer: 0.05 M Tris maleate buffer, pH 7.
‡ Number in parenthesis indicates the number of assays performed.
§ Controls were plates that were immediately overlaid with agar after exposure to bacteria without either THB or buffer washes.

TABLE V
Effect of Preexposure of the PFS to LTA (100 μg/ml) on the Adherence of S. sanguis

Treatment group	Adherence ratio 3 min*	15 min
Control	26.0±2.6	49.5±3.8
	(16)‡	(15)
LTA	17.1±1.4	34.5±4.1

* The time represents the number of minutes the PFS was preexposed to LTA.
‡ Indicates the number of determinations.

TABLE VI
Effect of Pretreatment of S. sanguis with Anti-LTA on Adherence to a PFS*

Type of serum	Adherence ratio	
	1/10 Dilution‡	1/100 Dilution
Anti-LTA	45.7±2.9	45.0±5.4
	(10)§	(10)
Control‖	52.8±3.2	45.4±7.4

* Sera obtained from rabbits immunized with LTA.
‡ Dilution of sera used in this study.
§ The number of assays performed.
‖ Pooled rabbit sera from nonimmunized rabbits.

in bacterial adherence in vivo. The largest difference between the adherence ratios of control and penicillin-pretreated cells was found in the 3-h samples (Table I) in which there was an actual decline in the number of adhering penicillin-pretreated cells from the 5-min time point. This suggests that the antibiotic-treated cells may only establish a "weak" attachment and subsequently detach from the valvular surface. The absence of a significant difference in valvular bacterial densities after 24 h is presumably due to the recovery and growth of the penicillin-treated bacteria once established on the surface.

Our in vitro studies also support the notion that penicillin interferes with the adherence of *S. sanguis*. Preincubation of *S. sanguis* with inhibitory but not bactericidal concentrations of penicillin (1 μg/ml, corresponding to ~30 × the MIC value) reduced adherence to the PFS while sub-MIC concentrations, 0.025 μg/ml, did not. This dependence on antibiotic dosage closely resembles the concentration dependence of penicillin-stimulated LTA release in *S. sanguis* (13).

The data shown in Table IV suggest that elution of streptococci already adherent to the PFS was unaffected by penicillin. In these experiments postincubation of already adhering bacterial cells with THB caused an increase in the number of cells adhering to the PFS (compare "untreated" cells to "control" cells in Table IV). This increase is probably due to continued replication, release and subsequent reattachment of cell progeny to the PFS. This is supported by the increase in the adherence ratio and by the wash ratios exceeding 1 (see Table IV and text).

The adherence ratios for the adherent *S. sanguis* exposed to penicillin were the same as the controls. This may be due to penicillin-induced inhibition of growth or suppression of adherence. The lack of a penicillin effect in the PFS incubated with Tris-buffer instead of growth medium (Table IV) may be compared with the results of biochemical experiments showing that the penicillin-stimulated release of LTA required active growth of the cells and would not take place in buffer (13).

Beachey and others demonstrated that when *S. pyogenes* was exposed to subinhibitory concentrations of penicillin, LTA was secreted from the cells into the medium and this was paralleled by a marked decrease in adherence to epithelial cells (5). Ramirez-Ronda and Gutierrez also reported recently that both ribitol teichoic acid and lipoteichoic acid blocked the adherence of *S. sanguis* to damaged heart valves in vitro (28).

Our in vivo and in vitro adherence data described here are consistent with the proposition that LTA may be an adhesin. Preexposure of the PFS to LTA extracted from the *S. sanguis* decreased adherence. Antisera directed against LTA was also able to reduce adherence when compared with pooled rabbit serum. Preincubation of the bacteria with antisera (1/10 dilution) significantly reduced adherence and incubation of penicillin-treated bacteria in LTA (1 mg/ml) restored the adherence to control levels. However, we did not observe decreased adherence by exposure of *S. sanguis* to sub-MIC concentrations of penicillin. The percent reduction in adherence caused by LTA in the present studies, 30% at 15 min, is < the 70% reported by Beachey (8). However, the assay used in the study reported here is different from that of Beachey, and consists of a matrix of varying constituents.

The *S. sanguis* strain used in this study is typical of the viridans streptococcal strains and has been well characterized in earlier in vitro studies as "tolerant" to the killing action of penicillin (11–13). Although not lysed by exposure to extremely high concentrations of penicillin, the strain does secrete LTA into the medium after exposure to penicillin at concentratiions above the MIC value (11–13).

This study demonstrates that inhibitory concentrations of penicillin reduce streptococcal adherence to surfaces resembling the nonbacterial thrombus first

TABLE VII
*Effect of Preincubation with LTA (1 mg/ml) of Penicillin-treated (1 μg/ml) S. sanguis on Adherence to a PFS**

Treatment group	Number of observations	Adherence ratio
Control‡	15	38.2±3.4
Penicillin treated	15	31.0±2.2
Penicillin and LTA treated	15	41.0±4.3

* Bacteria exposed to penicillin for 1 h were resuspended in buffer with or without LTA (1 mg/ml) for 0.5 h prior to exposure to the PFS for 15 min.
‡ The control samples were not treated with penicillin or LTA.

described by Angrist and Oka (26). The results are relevant to our understanding of the pathogenesis of subacute bacterial endocarditis and to some of the problems encountered in trying to prevent it. The studies suggest that loss of LTA from the bacterial cell may at least in part account for the reduction of bacterial adherence to host surfaces after exposure to penicillin. Adherence may constitute not only the initial step in the pathogenesis of endocarditis but may occur continually during the infection as part of a sequence of bacterial attachment, vegetation fragmentation, and bacterial reattachment to newly formed vegetation, a cycle that penicillin might interrupt.

The demonstration that tolerant bacteria exposed to penicillin are still capable of adhering to a valvular surface, and replicating after removal of penicillin, may be relevant for the potential failure of low concentrations of penicillin achieved after oral penicillin to prevent bacterial endocarditis.

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