Bacterial Adherence in the Pathogenesis of Endocarditis

INTERACTION OF BACTERIAL DEXTRAN, PLATELETS, AND FIBRIN

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ABSTRACT The role of dextran in the pathogenesis of bacterial endocarditis was investigated by studying the adherence of dextran producing oral streptococci to the constituents of nonbacterial thrombotic endocarditis (NBTE) in vitro and in vivo. The adherence of *Streptococcus sanguis* to fibrin and platelets was determined in an in vitro assay system simulating nonbacterial thrombotic endocarditis. Adherence was increased when the organisms were grown in sucrose-supplemented media (adherence ratio $\times 10^4$, 177 ± 6 in 5% sucrose vs. 140 ± 7 in 0.5% sucrose, $P < 0.001$), and decreased by incubating the organisms in dextranase (adherence ratio $\times 10^4$, 117 ± 16 , $P < 0.001$), an effect which was nullified by heat inactivating this enzyme (adherence ratio $\times 10^4$, 192 ± 7 , $P < 0.001$). The amount of dextran produced in broth by three different oral streptococci correlated directly with the adherence observed to fibrin and a fibrin-platelet matrix in vitro ($P < 0.001$). These organisms adhered more readily to a fibrin-platelet matrix than to fibrin alone (adherence ratio $\times 10^4$, 455 ± 30 vs. 177 ± 6 , respectively, $P < 0.001$).

The role of dextran formation was also examined in vivo in rabbits with preexisting NBTE. After injection of 10^7 *S. sanguis*, 12 of 17 animals developed endocarditis. In contrast, when the organisms were pretreated with dextranase (an enzyme that removes dextran from the bacterial cell surface), the same inoculum resulted in endocarditis in only 5 of 19 animals ($P < 0.05$). In addition, a fresh strain of *S. sanguis* that produced high levels of dextran ($1,220 \pm 50$ $\mu\text{g/ml}$) and adhered avidly to fibrin (adherence ratio $\times 10^4$, 220 ± 11) produced endocarditis in 12 of 18 rabbits after injection of 10^7 organisms. Another isolate of the same

strain that had been passed repeatedly in the laboratory produced less dextran (400 ± 30 $\mu\text{g/ml}$), adhered poorly to fibrin (adherence ratio $\times 10^4$, 140 ± 7), and produced endocarditis in only 3 of 14 rabbits under identical conditions ($P < 0.05$). This study demonstrates that dextran production is important in the adherence of oral streptococci to the constituents of NBTE and may play a role in the pathogenesis of bacterial endocarditis by oral streptococci.

INTRODUCTION

The precise conditions required for bacterial colonization of heart valves, the critical first step in the development of endocarditis, have never been specifically defined. Morphological studies with experimental models of endocarditis (1-4) have demonstrated that the initial colonization occurs in areas of endocardial trauma on platelet-fibrin deposits, so-called nonbacterial thrombotic endocarditis (NBTE)¹ (5-10). Angrist and Oka (5) have found similar lesions in pathological material from autopsies and have suggested that these deposits also serve as the nidus for bacterial colonization in humans. The factor(s) that influences the initial adherence of certain bacteria to these fibrin deposits has not been identified.

Dextran production has been identified as an important virulence factor in the adherence of oral streptococci to dental surfaces and the subsequent production of dental caries (11-15). Adherence is increased by growing the organisms in 5% sucrose-supplemented media (11, 16), a concentration which maximally stimulates dextran production (16). In addition, adherence is decreased by incubating the organisms with dex-

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¹Abbreviations used in this paper: AR, adherence ratio; BHIB, brain heart infusion broth; F-PFP, fibrin-platelet free plasma; F-PPP, fibrin-platelet poor plasma; F-PRP, fibrin-platelet rich plasma; NBTE, nonbacterial thrombotic endocarditis; PBS, phosphate-buffered saline; PFP, platelet-free plasma; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

tranase (17–19), an enzyme that removes dextran from the cell surface (20, 21). The virulence of dextran producing oral streptococci for caries production is caused by this attachment, a property which can be reversed by reducing cell-associated dextran. It is striking that the majority of cases of endocarditis are caused by these same dextran producing oral streptococci (22). Although the majority of organisms present in the bloodstream after dental manipulation do not produce dextran (23, 24), over 50% of the endocarditis cases are caused by dextran producing oral streptococci (23). In an analysis of 719 cases of streptococcal bacteremia, Parker and Ball (22) identified 241 cases of endocarditis. The four organisms with the greatest propensity to produce endocarditis were dextran producing oral streptococci. Therefore, the relative ability of certain oral streptococci to produce endocarditis may reflect their ability to produce dextran, i.e., dextran production may be a virulence factor in this disease. The purpose of these studies was to investigate the importance of dextran production in the pathogenesis of bacterial endocarditis by studying its influence on the adherence of oral streptococci to the components of NBTE (i.e., platelets and fibrin) in vitro and in producing endocarditis in vivo.

METHODS

In vitro methods

Dextran producing oral streptococci grown in either 0, 0.5, or 5% sucrose and with or without dextranase were exposed to a fibrin surface in vitro. All experiments were done in platelet-free and platelet-rich plasma according to the following specific methods.

Preparation of bacteria. *Streptococcus sanguis* M-5 (obtained from Dr. R. Gibbons, Forsythe Dental Institute, Boston, Mass.) was grown in brain heart infusion broth (BHIB) with phosphate buffer (pH = 6) alone or supplemented with either 0.5% or 5% sucrose (Sigma Chemical Co., St. Louis, Mo., grade I). In addition, organisms were grown in a chemically defined medium (FMC) for some experiments. FMC contains 1% glucose, 0.5% casamino acid solution (Difco, Difco Laboratories, Detroit, Mich.), and the following salts (per 10 l): NaCl 20 g, $K_2HPO_4 \cdot 3H_2O$ 40 g, KH_2PO_4 20 g, K_2CO_3 10 g, $MnSO_4$ 150 mg, $MgSO_4$ 1.2 g. The final pH was 7.2. An 18-h culture was filtered through an 8- μ m Millipore filter (Millipore Corporation, Bedford, Mass.) twice to remove aggregated forms (verified by observations of only single organisms in a Petroff-Hauser chamber), centrifuged at 3,000 rpm for 15 min, washed twice in phosphate-buffered saline (PBS), and quantified by optical density measurements to ensure an inoculum size of 10^3 (Gilford spectrophotometer 2400, Gilford Instrument Laboratories, Oberlin, Ohio). An isolate of *S. sanguis* M-5 which had been repeatedly subcultured in the laboratory (*S. sanguis*₁), a fresh isolate of the same *S. sanguis* strain (designated *S. sanguis*₂), *Streptococcus salivarius*, *Streptococcus mutans* GS5, and *S. mutans* 6715 (both obtained from Dr. H. Kuramitsu, Northwestern University, Chicago, Ill.) were prepared in an identical fashion.

Preparation of experimental surfaces. The fibrin layer was prepared as the classical fibrin plate-plasminogen assay (26–

28) with modifications as follows: bovine fibrinogen (Sigma Chemical Co.) was dissolved in PBS (Ca, Mg free) at 10 mg/ml and 0.2 ml evenly spread onto standard 60 × 15-mm tissue culture dishes (Falcon Plastics, Division of Bioquest, Oxnard, Calif.) with a glass stirring rod. 0.5 ml of bovine thrombin (Thrombin, topical, 1,000 National Institutes of Health (NIH) U/vial, Parke-Davis, Detroit, Mich.) at 500 U/cm³ was added, and the plates incubated overnight. The surface was washed five times with PBS and used as below.

Preparation of fibrin-platelet matrix. To simulate more closely the NBTE seen in vivo, human blood was collected (4.5 ml) in 7 cm³ tubes containing 0.5 cm³ of 3.8% Na citrate. Donors had not ingested aspirin for the previous 10 days. This was centrifuged at 2,000 rpm for 3 min; the supernate was removed and designated PRP. The remainder was centrifuged at 3,000 rpm for 15 min; the supernate was removed and designated platelet-poor plasma (PPP). The platelet counts were enumerated manually by phase-contrast microscopy; PRP was adjusted to a count of 300,000/mm³, and the mean platelet count for PPP was 2,890/mm³ (range 1,000/mm³–3,800 per mm³, *n* = 67). Platelet suspensions were kept in silicone-coated glassware. PPP was also filtered twice sequentially through 8-, 1.2-, and 0.45- μ m Millipore filters; the product was designated PFP. The platelet count of PFP was uniformly 0/mm³ (*n* = 26). 1 ml of PRP, PPP, or PFP was combined in the tissue culture dishes with 0.4 ml bovine thrombin (Thrombin, topical, 1,000 National Institutes of Health U/vial, Parke-Davis) at 500 U/cm³ and 0.4 ml 0.2 M CaCl₂ and incubated for 30 min at 37°C. All reagents were filter-sterilized (0.22 μ m) twice. Evenly spread adherent clots containing fibrin and platelets in two concentrations as well as fibrin alone were thus obtained (designated F-PRP, F-PPP, and F-PFP) and exposed to organisms as below.

Determination of adherence. The bacterial inoculum, in a volume of 5 ml of PBS, was poured into the tissue culture dishes containing either the layer of fibrin or fibrin plus platelets and agitated for 15 min at 37°C in a shaking incubator (Dubnoff metabolic 66799, Precision Scientific Group, Chicago, Ill.), oscillating at 120 cycle/min. After exposure, the supernate was removed and titered on duplicate blood agar pour plates. The surface was then washed with PBS three times sequentially on a rotatory shaker for five min per wash (Yankee variable speed rotator, Clay-Adams Division, Becton, Dickinson & Co., New York). Each wash (volume = 5 ml) was removed and titered on duplicate blood agar pour plates. After washing, the surface was overlain with trypticase soy agar (Baltimore Biological Laboratories, Baltimore, Md.) containing 0.5% defibrinated sheep blood and incubated for 24 h at 37°C. The adherent organisms were enumerated by colony count; the adherence ratio (AR) is defined as the number of organisms adherent to the surface (colony forming units) divided by the number of organisms in the initial inoculum (colony forming units). All statistical analysis was done on paired data utilizing Student's *t* test.

Influence of dextran on bacterial adherence. Bacterial adherence to fibrin and fibrin-platelet surfaces was determined: (a) after 18 h incubation of *S. sanguis* in BHIB supplemented with 0, 0.5, or 5% sucrose; (b) after 18 h incubation of *S. sanguis* with dextranase or heat-inactivated dextranase; (c) for *S. sanguis* grown in BHIB (0% sucrose) after the addition of exogenous soluble dextran; (d) for three oral streptococci that produce different amounts of extracellular dextran, and one organism (*S. salivarius*) that produces only levan.

In vitro dextranase studies. A 50% pure preparation of dextranase (obtained from Dr. Thomas Stoudt, Merck, Sharp, and Dohme, Rahway, N. J.) with an activity of 14,400 U/mg was dissolved in brain heart infusion broth (BHIB) (Difco, Difco Laboratories) with or without supplemental sucrose and

buffered to pH 6.0 with 0.2 M PO_4 buffer yielding a dextranase activity of 57 U/ml. Stock dextranase solutions in phosphate-buffered saline (PBS) were used within 2 wk of preparation. All organisms were incubated for 18 h in BHIB plus 0, 0.5, or 5% sucrose buffered to pH 6.0 with either dextranase or heat-inactivated (65°C for 30 min) dextranase added. The adherence ratios for these two groups were compared to control organisms grown in sucrose-supplemented BHIB without dextranase. The presence of dextranase in the incubating medium did not affect bacterial viability or growth rates because titers reached in broth were equivalent to controls.

Exogenous dextran studies. *S. sanguis* was grown in BHIB (without supplemental sucrose) alone, or with the addition of dextranase (see above). Dextran T-2000 (Sigma Chemical Co.) was dissolved in PBS at 5 mg/ml and added in equal volume to the standard bacterial suspensions (2.5 mg/ml final concentration). The adherence of these organisms was compared to controls. The addition of exogenous dextran (or equal volumes of PBS) did not adversely affect bacterial viability.

Determination of dextran production in broth. The amount of dextran produced in broth by the oral streptococci was determined, with modifications, by the method of Gibbons and Barghart (11). Organisms were grown in BHIB (phosphate buffered to pH 6) supplemented with sucrose and/or dextranase (see above) for 48 h. 10 ml was blended in a Sorvall omnimixer (DuPont Instrument Products Division, Sorvall Operations, Wilmington, Del.) for 30 s at 4°C to remove cell-associated dextran. The organisms were removed by centrifugation at 1,000 rpm for 15 min. The polysaccharide in the supernate was precipitated with the addition of 1.5 vol 95% ethanol, and centrifuged at 3,000 rpm for 15 min. The pellet was washed twice in 65% ethanol, recentrifuged as above, rinsed in distilled water, and again centrifuged at 3,000 rpm for 15 min. Residual protein (less than 2%) was removed by the addition of TCA to a 10% concentration and subsequently dialyzed overnight. The polysaccharide was then reprecipitated in 2 vol of 95% ethanol, centrifuged at 3,000 rpm $\times$ 15 min, lyophilized, and weighed. The results are expressed as micrograms of dextran per milliliter of starting broth and all values are corrected for control values (polysaccharide determined for uninoculated BHIB under identical conditions).

Scanning electronmicroscopy of fibrin surfaces. The various surfaces were prepared as described and exposed to unfiltered *S. sanguis* in the standard manner. The surface was then fixed in 2.5% glutaraldehyde (Fisher Scientific Co., Fair Lawn, N. J., biological grade) with phosphate buffer and dried in sequential washings of 40, 60, 80, and 100% ethanol in distilled water. The plates were cut into $\sim 3 \times 3$ mm squares and mounted on grids with silver paint (G. C. Electronics Co., Rockford, Ill.). A 300–500 Å gold palladium coating was applied (Hummer Technics, Alexandria, Va.), and the surface was examined in the scanning electron microscope (Cambridge Stereoscan, Mark IIA, Cambridge Instruments, England).

In vivo methods

Production of endocarditis. Left-sided bacterial endocarditis was produced in 68 2-kg albino New Zealand rabbits by a modification of methods described previously (2, 4). A polyethylene catheter (Intramedic, Clay-Adams, PE-90) was introduced through the carotid artery and across the aortic valve. Correct placement was indicated both by the absence of blood return and the presence of a characteristic movement of the catheter with systole. The catheter was flushed with normal saline and clamped in place for 5 min. This procedure produces trauma to the valvular endocardium and uni-

formly results in the deposition of platelets and fibrin on the surface (4). The catheter was withdrawn 1 cm and a single inoculum of 10^7 organisms (The exact number of organisms was derived by serial dilutions of an 18-h culture and verified by quantitative culture by split specimens on agar.) was injected through it. The catheter was then removed entirely and the wound closed. The presence or absence of endocarditis was confirmed by quantitative blood and valve cultures at autopsy 48 h after catheterization (25). Serial dilutions of aortic valve cusps were made after wide excision and homogenization. To ensure uniformity of technique, all rabbits were done by the same investigator (J. A. V.) over the same time period.

In vivo dextranase study. To assess the effect of dextranase pretreatment upon the ability of the streptococci to initiate bacterial endocarditis, dextran producing *S. sanguis*, grown in BHIB with 0.5% sucrose with and without dextranase as previously described, were washed twice in normal saline and 10^7 organisms injected into 36 rabbits which had sustained 5 min of catheter-induced aortic valve trauma. The presence of endocarditis was determined after 48 h (see above).

Relationship of dextran production to endocarditis production in vivo. 32 additional rabbits were catheterized as above and 10^7 organisms were injected into the left ventricle. The first group received *S. sanguis*₁, a strain passed frequently in the laboratory (with low dextran production) and the second group received *S. sanguis*₂, a fresh isolate of the same strain (with high dextran production in broth). All organisms were grown in BHIB with 0.5% sucrose. The relative propensity for these two isolates to produce endocarditis was ascertained when the animals were sacrificed 48 h after inoculation, as above.

Scanning electronmicroscopy. Two rabbits were catheterized as above and sacrificed at 48 h. The aortic valves were removed, fixed in 2.5% glutaraldehyde, sequentially dried in ethanol, mounted on grids, coated with 300 Å gold palladium, and examined.

RESULTS

In vitro bacterial adherence

STANDARDIZATION OF IN VITRO ADHERENCE TECHNIQUES

The number of bacteria in the incubating solution that adhere to the fibrin layer could be influenced by multiple factors. Initial studies were therefore conducted to identify potential artifacts, firmly standardize the system, and determine the reproducibility of the methods. The influence of the following factors on bacterial adherence was determined.

Relationship of adherence to initial titer of bacteria in inoculating medium. The number of organisms adherent to the surface was a constant proportion of the initial inoculum titer. Over a 1,000-fold range, linearity was observed between the AR and the initial inoculum titer when the surface was exposed to a constant volume of 5 ml. For inocula of 5×10^2 to 7×10^5 , the AR ranged from 143 to 210 (mean = 182 ± 7 , $n = 22$); these values did not differ significantly from each other ($P > 0.05$). A standard inoculum of 10^3 was chosen for all subsequent experiments.

Relationship of adherence to volume of inoculum.

Because smaller inoculum volumes could increase the contact between the organism and the surface under investigation, the AR was determined for equal bacterial titers but in varying inoculum volumes (Fig. 1). The AR is increased at the smaller volumes studied (1 and 2.5 ml) whereas at 5, 6, and 7 ml the AR was relatively constant. This phenomenon was demonstrated for all surfaces studied. A standard volume of 5 ml was therefore chosen for all subsequent experiments.

Relationship of adherence to time of incubation. The effect of varying the time of incubation for the organism and surface under investigation on the AR observed was determined (Fig. 2). Substantial adherence is observed as early as 1 min after exposing the surface to the inoculum and the AR increased with time, reaching a plateau at 15–30 min. This was observed with both fibrin and a fibrin-platelet matrix. An incubation time of 15 min was chosen for all experiments.

Relationship of organism removal to number of washes. The effect of the number of sequential 5-min washes on the removal of organisms adherent to the surface was evaluated. The first wash removed a considerable number of organisms but this was less than 1% of the initial inoculum ($n = 27$). Two additional washes removed 0.1% and no further removal was observed when more than a total of three washes were used ($n = 18$). A standard number of three washes was utilized in all experiments.

Role of bacterial clumping. Because the number of adherent organisms was determined by counting bacterial colonies after 24 h of incubation, aggregated masses or multiple organisms might result in only one colony forming unit when enumerated after incubation. This was minimized by filtration and observing single

FIGURE 2 Effect of incubation time on adherence of *S. sanguis* to fibrin or a fibrin-platelet matrix (F-PRP). Organisms were grown in 5% sucrose-supplemented BHIB.

unaggregated bacteria in the standard bacterial suspensions. In addition, the following ratio was calculated for all experiments: (number of bacteria in initial inoculum – number adherent to surface)/(number of bacteria eluted in supernate after exposure). If all colonies result from a single organism, this ratio should equal 1. The actual value for 226 observations is 1.04. Considering the number of organisms removed in the washes, this confirms that virtually every colony on the surface results from a single adherent organism.

Susceptibility of oral streptococci to serum. Because traces of serum may be present in all surfaces, the effect of serum on bacterial viability was investigated. All organisms obtained equal titers ($\geq 10^8$) at 18 h whether grown in normal serum or heat-inactivated (57°C for 30 min) serum. Thus, these organisms were not “serum sensitive.”

Specificity of adherence to fibrin layer. Because *S. sanguis* may also adhere to the plastic tissue culture dishes as well as the fibrin, the bacterial colonies could represent bacteria that had penetrated through the thin fibrin layer (<0.3 mm) and adhered to plastic. The fibrin layer was removed with streptokinase and specificity of attachment determined (Table I). Streptokinase-streptodornase (Varidase, Lederle Laboratories, American Cyanamid, Co., Pearl River, N. Y.) was reconstituted in 2 ml of PBS and filter sterilized through an 0.22 μ m Millipore filter twice. The fibrin layer was exposed to *S. sanguis* as described, and overlain with 2 ml of this solution (10,000 U/ml), and placed in a shaking incubator for 4 h at 150 cycle/min at 37°C. Streptokinase acts by activating plasminogen and this procedure effectively dissolved the fibrin layer. The mixture was decanted to a fresh tissue culture plate, overlain with agar, incubated for 24 h at 37°C, and enumerated by colony count. The enzyme-treated “orig-

FIGURE 1 Effect of inoculum volume on adherence of *S. sanguis* to fibrin.

TABLE I
Effect of Streptokinase on Adherence of
Streptococcus Sanguis

Surface	AR $\times 10^4$ mean $\pm$ SEM	$\frac{y-x}{y}$, mean $\pm$ SEM
<i>Fibrin</i>		
$x = \frac{\text{adhere original plate}}{\text{initial titer}} \text{ (} n = 11 \text{)}$	29 $\pm$ 3*	+0.838 $\pm$ 0.004*
$y = \frac{\text{adhere to new plate}}{\text{initial titer}} \text{ (} n = 11 \text{)}$	147 $\pm$ 9*	
<i>Plastic</i>		
$x = \frac{\text{adhere original plate}}{\text{initial titer}} \text{ (} n = 11 \text{)}$	200 $\pm$ 7*	-0.851 $\pm$ 0.003*
$y = \frac{\text{adhere to new plate}}{\text{initial titer}} \text{ (} n = 11 \text{)}$	36 $\pm$ 4*	

* $P < 0.001$.

inal" plate was treated in the same manner. Plastic tissue culture dishes without a fibrin layer were also exposed to *S. sanguis* and treated with enzymes as described. These studies demonstrate that 83% of the adherent organisms were removed when the fibrin layer was treated with streptokinase (Table I), whereas streptokinase was ineffective in removing organisms from the plastic plates without fibrin. If a ratio denoting (AR new plate - AR original plate)/AR new plate is derived, this value should equal +1 for fibrin and -1 for plastic. The actual values are +0.838 and -0.851, respectively. Thus, over 80% of the adherent bacteria were specifically attached to the fibrin.

EFFECT OF SUCROSE CONCENTRATIONS

The *S. sanguis*₁ adhered better to all fibrin and fibrin-platelet surfaces when grown in media containing high sucrose concentrations (5%) than when grown in low sucrose concentrations (0.5%) ($P < 0.001$) or no sucrose ($P < 0.001$) (Table II). For example, the AR of the organism to the F-PRP matrix is 455 $\pm$ 30 after growth in 5% sucrose; but decreased to 248 $\pm$ 10 when grown in 0.5% sucrose ($P < 0.001$) and further decreased to 190 $\pm$ 4 when grown in BHIB without supplemental sucrose. The addition of only 0.5% sucrose to BHIB always increased the AR over organisms grown in BHIB alone ($P < 0.001$, all four surfaces). Furthermore, the AR observed for *S. sanguis* grown in BHIB are equivalent to those obtained after growth in the chemically defined medium (FMC).

EFFECT OF DEXTRAN TREATMENT

Incubation of *S. sanguis*₁ in dextranase uniformly decreased adherence to the four experimental surfaces (Table II) when organisms were grown in either high or low concentrations of sucrose ($P < 0.001$). In each case, the dextranase effect on adherence ($P < 0.001$) was nullified by heat inactivating this enzyme. Heat-inactivated dextranase-treated organisms did not differ significantly from controls (P range 0.11-0.36). The 18-h incubation of bacteria in the presence of dextranase did not inhibit viability or growth rates; equal bacterial titers were reached ($\geq 10^8$) for all three experimental conditions. Dextranase treatment did not change the AR for *S. sanguis* grown in the absence of sucrose. When grown in BHIB (0% sucrose) the adherence ratios for the F-PRP and F-PPP were 190 $\pm$ 4 and 213 $\pm$ 5, respectively. The values were 194 $\pm$ 4 in

TABLE II
Bacterial Adherence to Fibrin and Fibrin-Platelet Matrices

Strain/treatment	Sucrose	AR $\times 10^4$, mean $\pm$ SEM			
		Fibrin	F-PRP	F-PPP	F-PFP
	%				
<i>S. sanguis</i> ($n = 15$)	5.0	177 $\pm$ 6	455 $\pm$ 30	684 $\pm$ 56	283 $\pm$ 12
<i>S. sanguis</i> /dextranase ($n = 14$)	5.0	117 $\pm$ 16	220 $\pm$ 17	293 $\pm$ 22	80 $\pm$ 4
<i>S. sanguis</i> /heat-inactivated dextranase ($n = 13$)	5.0	192 $\pm$ 7	470 $\pm$ 15	747 $\pm$ 30	292 $\pm$ 12
<i>S. sanguis</i> ($n = 11$)	0.5	140 $\pm$ 7	248 $\pm$ 10	391 $\pm$ 16	152 $\pm$ 4
<i>S. sanguis</i> /dextranase ($n = 9$)	0.5	71 $\pm$ 7	99 $\pm$ 5	122 $\pm$ 8	48 $\pm$ 2
<i>S. sanguis</i> /heat-inactivated dextranase ($n = 11$)	0.5	153 $\pm$ 9	294 $\pm$ 22	411 $\pm$ 7	157 $\pm$ 3
<i>S. sanguis</i> (BHIB) ($n = 22$)	0	102 $\pm$ 4	190 $\pm$ 4	213 $\pm$ 5	109 $\pm$ 4
<i>S. sanguis</i> (FMC) ($n = 11$)	0	104 $\pm$ 3	191 $\pm$ 2	204 $\pm$ 5	111 $\pm$ 4
<i>S. sanguis</i> (BHIB)/exogenous dextran ($n = 12$)	0	161 $\pm$ 6	291 $\pm$ 12	506 $\pm$ 14	258 $\pm$ 7

FMC, chemically defined medium (see Methods).

each instance after exposure to the enzyme ($P = 0.38$) ($n = 8$). Thus, selective removal of the extracellular dextran with enzyme treatment reduced adherence of the *S. sanguis* to fibrin and platelet-fibrin matrices.

EFFECT OF EXOGENOUS SOLUBLE DEXTRAN

The role of dextran as a possible ligand between oral streptococci and NBTE was further examined by adding exogenous soluble dextran to *S. sanguis* bacterial suspensions after growth in BHIB without supplemental sucrose (Table II). For each surface, exogenous dextran increased the AR to levels equivalent to medium-high sucrose concentrations ($P < 0.001$).

EFFECT OF PLATELETS

The presence of platelets increased the AR for *S. sanguis*₁ over values observed on fibrin alone (Table II). The mean AR to bovine fibrin was 177 ± 6 ; and increased to 455 ± 30 on the fibrin-platelet rich matrix ($P < 0.001$). The AR was greater for the fibrin-platelet surface made from PPP than the fibrin F-PRP surface ($P < 0.001$). Both platelet-fibrin surfaces exhibited greater adherence ratios than the fibrin plus PFP (283 ± 12) ($P < 0.001$). Results were similar with a fresh isolate of the M-5 strain (*S. sanguis*₂). When the organism was grown in BHIB and 0.5% sucrose, the AR observed on bovine fibrin was 220 ± 11 . This was increased on both F-PRP and F-PPP to 533 ± 26 and 499 ± 14 , respectively, and both were greater than on fibrin alone ($P < 0.001$). With this organism there was no difference in adherence for fibrin-PRP versus fibrin-PPP ($P = 0.198$). Thus, the presence of platelets increased adherence, an effect that was not dependent on platelet concentration over the range studied (3,000–300,000 platelets/mm³).

RELATIONSHIP OF ADHERENCE TO DEXTRAN FORMATION IN BROTH

The amount of dextran produced in broth directly correlated with the adherence ratios (Table III). *S. sanguis*₁ produced 404 ± 26 μ g of dextran per ml of broth when supplemented with 0.5% sucrose and 799 ± 49 μ g/ml when the organism was grown in 5% sucrose ($P < 0.001$). Only 142 ± 20 μ g/ml was produced when dextranase was added to the incubating media ($P < 0.001$). The amount of dextran produced when *S. sanguis*₁ was incubated in BHIB with heat-inactivated dextranase did not differ from control values ($P = 0.38$). In all circumstances, the amount of dextran produced in broth correlated directly with the AR observed for all surfaces ($P < 0.001$). The adherence ratio observed for three other oral streptococci also directly correlated with the amount of dextran produced by the organism in broth ($P < 0.001$) (Table III). In addition, the AR

TABLE III
Relationship of Adherence to Dextran-Production

Organism/treatment	Sucrose	Dextran-production	AR $\times 10^4$ mean $\pm$ SEM (to fibrin)
	%	μ g/ml of broth mean $\pm$ SEM	
<i>S. sanguis</i> ₁	0.5	404 ± 26	140 ± 7
<i>S. sanguis</i> ₁ /dextranase	0.5	142 ± 20	71 ± 7
<i>S. sanguis</i> ₁ /heat-inactivated dextranase	0.5	431 ± 29	153 ± 9
<i>S. sanguis</i> ₁	5.0	799 ± 49	177 ± 6
<i>S. sanguis</i> ₁ /dextranase	5.0	181 ± 29	117 ± 16
<i>S. sanguis</i> ₁ /heat-inactivated dextranase	5.0	826 ± 37	192 ± 7
<i>S. sanguis</i> ₂	0.5	$1,215 \pm 53$	220 ± 11
<i>S. mutans</i> GS5	5.0	$4,762 \pm 89$	376 ± 9
<i>S. mutans</i> 6715	5.0	$5,858 \pm 109$	480 ± 21

for *S. salivarius* (which produces only levan) was 94 ± 7 , a value significantly lower ($P < 0.001$) than all the dextran producing strains.

SCANNING ELECTRONMICROSCOPY

Examination of the F-PRP surface with scanning electronmicroscopy demonstrated the coarse fibrin background (Fig. 3). Streptococci are visualized on the surface interspersed among larger, aggregated platelets. Platelets and platelet-aggregates were identified on F-PPP, but in reduced numbers, and were absent from F-PFP and the free fibrin surfaces. The streptococci were easily identified in areas of platelet aggregation but were infrequently visualized in areas containing fibrin alone. The surface of a traumatized rabbit aortic valve (Fig. 4) also exhibited the coarse fibrin meshwork with numerous platelets attached to the matrix. The close resemblance of this in vivo surface to the F-PRP prepared in vitro is apparent.

In vivo studies

EFFECT OF DEXTRANASE INCUBATION OF *S. SANGUIS* IN EXPERIMENTAL ENDOCARDITIS (TABLE IV)

When 10^7 organisms (grown in BHIB and 0.5% sucrose) of a fresh isolate of *S. sanguis* were injected into rabbits with pre-existing NBTE, 12 of 17 animals developed endocarditis. In contrast, when the organisms were preincubated in dextranase to remove the dextran from the cell surface, only five of 19 animals developed endocarditis under identical conditions ($\chi^2 = 5.39$, $0.02 < P < 0.05$). Thus, dextranase treatment

FIGURE 3 Scanning electronmicrograph of the F-PRP surface exposed to *S. sanguis*. F = fibrin; P = platelet; S = *S. sanguis*. ($\times 9,030$; Bar = 5 μm)

appeared to alter the ability of dextran producing streptococci to colonize traumatized aortic valves and produce endocarditis in rabbits. Dextranase did not interfere with growth or electronmicroscopic characteristics of the organism; both treated and untreated streptococci grew to identical titers (10^8 colony forming units/ml) after 18 h of incubation.

EFFECT OF DEXTRAN PRODUCTION ON ENDOCARDITIS PRODUCTION BY *S. SANGUIS* (TABLE V)

Dextran production in broth and the corresponding adherence ratio was directly predictive of the ability of the organism to produce endocarditis in rabbits. *S. sanguis*₁ produces low levels of dextran, demonstrates a low AR, and endocarditis was found in only three of 14 rabbits when sacrificed 48 h after inoculation. In contrast, *S. sanguis*₂ produces more dextran, adheres to fibrin more avidly in vitro, and produced endocarditis in 12 of 18 rabbits under identical conditions. This difference is statistically significant ($\chi^2 = 4.78$, $0.02 < P < 0.05$).

DISCUSSION

The development of bacterial endocarditis requires a unique interaction of a microorganism with the tissues of the host. In this study, we have shown that dextran production by *S. sanguis* is a factor that influences this organism's ability to adhere to an in vitro surface simulating NBTE and to produce endocarditis on traumatized aortic valves in vivo. The presence of platelets in a fibrin matrix increases adherence of *S. sanguis* over that observed with fibrin alone, and platelets are easily identified on the surface of the damaged valve.

The initial event in the production of bacterial endocarditis must be the colonization of receptive valve surfaces by bacteria. Certain species of bacteria seem to possess specific characteristics that enable them to avidly adhere to these surfaces. The relative propensity for a small select group of microorganisms to cause the majority of cases of bacterial endocarditis (6, 30) has never been explained, but bacterial adherence to the valve surface may be a crucial virulence factor. In a rabbit model of endocarditis, Valone et al. (31) have demonstrated that *Streptococcus aureus* and viri-

FIGURE 4 Scanning electronmicrograph of traumatized rabbit aortic valve (NBTE). F = fibrin; P = platelets ($\times 4,360$)

dans streptococci produced endocarditis more readily than *Escherichia coli* (infectious dose for 50% of animals = $10^{3.75 \pm 0.60}$ and $10^{4.67 \pm 0.52}$ vs. $10^{6.29 \pm 0.58}$, respectively $P < 0.05$). Gould et al. demonstrated that viridans streptococci and *S. aureus* adhered better than *E. coli* to punch biopsies of canine aortic valve leaflets in vitro (32). These two studies suggest that bacterial adherence in vitro may correlate with the relative predilection of certain organisms to produce endocarditis in vivo and also with the relative frequency of the disease in man. The present study extends these observations to the most common causative agent of endocarditis, *S. sanguis*, (23, 24) and demonstrates that factors that affect adherence to fibrin and platelets also influence the endocarditis producing ability in vivo. The importance of the fibrin-platelet matrix, so-called NBTE, as

the critical receptive host tissue for colonization by bacteria has been suggested by Angrist and Oka (5) and repeatedly confirmed in the rabbit model (3, 4, 9).

The role of bacterial adherence in colonization and resultant infection is not unique to bacterial endocarditis but has been defined in numerous infections, including gonorrhea (33, 34), bovine conjunctivitis (35), piglet (36–38), calf (39) and human diarrhea (40), and streptococcal pharyngitis (41).

The ability of oral streptococci to adhere to smooth surfaces and their cariogenic potential have also been

TABLE IV
Effect of Dextranase Treatment of *S. sanguis* on the Development of Endocarditis

Group	No. with endocarditis/total no.
Control organisms	12/17*
Organisms grown in dextranase	5/19*

* $0.02 < P < 0.05$.

TABLE V
Effect of Dextran-Production on Development of Endocarditis

Organism	Dextran-production $\mu\text{g/ml}$ broth mean $\pm$ SEM (n = 4)	Adherence ratio $\times 10^4$ (to fibrin) mean $\pm$ SEM (n = 9)	No. with endocarditis/ total no.
<i>S. sanguis</i> ₁	404 $\pm$ 26*	140 $\pm$ 7†	3/14§
<i>S. sanguis</i> ₂	1,215 $\pm$ 53*	220 $\pm$ 11†	12/18§

* $P < 0.001$.

† $P < 0.001$.

§ $P < 0.05$.

shown to depend on the production of extracellular dextran (11–13). Dental plaque formation is decreased by prior treatment with dextranase (17–19, 42), by growth in low sucrose supplemented media (11), by mutants lacking the ability to synthesize dextran (44), and by antibody directed toward whole organisms or glucosyltransferases (44, 45), the enzymes responsible for dextran synthesis.

In the *in vivo* studies reported here, the propensity of *S. sanguis* for endocarditis production was reduced by incubating the organism with dextranase, suggesting that extracellular dextran may also influence the adherence of *S. sanguis* to traumatized aortic valves (with a fibrin-platelet surface) *in vivo*. In addition, the amount of dextran produced in broth is directly predictive of the potential of endocarditis producing organisms. However, a clear delineation of the critical factor(s) implicit in this interaction between the host tissue surface and the infecting microorganism is impossible in an *in vivo* model. The *in vitro* assay system described allows a systematic separation of these factor(s) for study. We have demonstrated that this system is reliable, reproducible, and consistent under a wide variety of experimental conditions.

Studies designed to standardize the *in vitro* assay system demonstrated that the adherence of *S. sanguis* to a surface simulating NBTE is a constant proportion of the initial inoculum titer, but increased with lower inoculum volumes and longer duration of exposure. These results confirm and extend those of Gould et al. (32). Similar conditions influence the development of endocarditis *in vivo*. Because a critical infectious dose for 50% of animals can be determined for each organism, the larger the infecting inoculum the greater the chance of producing disease. The concentration of the incubating inoculum (10^9 /ml) used in this *in vitro* system is similar to the peak concentration of organisms achieved in the bloodstream after various dental and urological procedures (46). Likewise, endocarditis has been induced by procedures that block reticuloendothelial clearance of bacteria which prolong the duration of bacteremia and increase the time of exposure to the valve surface (47). The adherence of bacteria to the fibrin surfaces in these experiments occurred rapidly. The time of exposure (15 min) was well within the duration of bacteremia noted in patients and experimental animals after a procedure or single intravenous injection (48). Although the amount of fibrin present in the *in vitro* assay system is likely to exceed that present *in vivo*, the similarity of the two surfaces is confirmed by scanning electron microscopy.

Vigorous sequential washings of the fibrin surface after exposure failed to dislodge substantial numbers of the adherent organisms. The strength of this bond is likely of critical importance on the surface of heart valves *in vivo* where high velocity blood flow would

tend to disperse organisms that were loosely adherent to the surface. Thus, once initial attachment occurs, the more tightly adherent strains would possess a selective advantage allowing them to resist removal and persist on the valve. Further platelet-fibrin deposition then occurs resulting in the mature bacterial vegetation. The ability of certain streptococci to induce platelet aggregation and the release reaction (49, 50) may thus also contribute to their survival on the valve surface.

An important role for dextran in the adherence of *S. sanguis* *in vitro* is demonstrated by an increase in adherence if the organisms are grown in 5% > 0.5% > 0% sucrose-supplemented media and by a decrease in the adherence ratio when the organisms are incubated with dextranase, an effect which was completely nullified by heat inactivating this enzyme. The addition of exogenous dextran to organisms grown in control (0% sucrose) media increased adherence to values equivalent to those found after sucrose supplementation. In addition, the amount of dextran produced in broth was directly predictive of the AR observed for four oral streptococci. The presence of the extracellular polysaccharides on the cell surface of the bacteria thus increase adherence of the oral streptococci to fibrin and platelet-fibrin matrices similar to its role in adherence to enamel and other dental surfaces. The parallel experiments *in vivo* demonstrate that the presence of extracellular dextran similarly influenced the production of bacterial endocarditis.

Bacterial adherence is also influenced by the characteristics of the host surface. In these studies the presence of platelets markedly increased the bacterial adherence to the fibrin surface. *In vivo* the receptive host tissue (NBTE) is initially composed of fibrin plus platelets; a surface that would promote ideal conditions for adherence of these dextran producing strains (3). Platelets potentiate bacterial adherence to NBTE even at low concentrations. In these studies, adherence was increased if platelets were present in the matrix but did not increase with higher platelet concentrations. Studies in rabbits pretreated with antiplatelet drugs, such as aspirin (29) or aspirin plus dipyridamole² have demonstrated the inability of these drugs to increase the infectious dose for 50% of animals required to produce the disease. These results suggest that a low but critical concentration of platelets may be necessary to maximally influence adherence, and antiplatelet drugs are unlikely to completely inhibit platelet aggregation on the fibrin matrix. In aspirin treated rabbits, NBTE develops, and platelets are demonstrated by light microscopy and electron microscopy (29).

The AR observed in this study are higher than those obtained by Gould et al. (32), however, we have meas-

² Valone, J. A., and M. A. Sande. Unpublished observations.

ured adherence to a fibrin or fibrin-platelet surface, and not to normal endothelium. Recently it was shown that dextran production by four strains of *S. mutans* increased their ability to adhere to damaged canine aortic valves (coated with a fibrin-platelet matrix) but not to normal, undamaged, endothelium in vitro (51). Dextran producing strains adhered four to five times more avidly than nondextran producing strains. The adherence ratio for these four dextran producing strains of *S. mutans* ranged from 438 to 510, in close agreement with the adherence ratio of 455 reported in this study for *S. sanguis* to a F-PRP matrix. Thus, the ability of *S. sanguis* to adhere to traumatized valves with a fibrin-platelet nidus is greater than to undamaged endothelium.

These in vitro studies correlate closely with the in vivo observations, and contribute to our understanding of the complex pathogenesis of endocarditis.

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