

Influence of Preformed Antibody on Experimental *Streptococcus sanguis* Endocarditis

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The influence of preformed, anti-whole organism antibody on the development of *Streptococcus sanguis* endocarditis was examined in both in vivo and in vitro systems. Antibody prevented, rather than potentiated, endocarditis in rabbits. The infectious dose in 30 control animals was $10^{6.5 \pm 0.33}$ (mean \pm standard deviation); this increased to $10^{7.71 \pm 0.05}$ in 36 immunized animals ($P < 0.01$). No differences in bacterial clearance mechanisms were apparent between groups. Antibody also prevented the adherence of *S. sanguis* to the constituents of nonbacterial thrombotic endocarditis (fibrin and platelets) in vitro. When preincubated in high-titer antisera, adherence of *S. sanguis* was reduced compared with controls (adherence ratio mean \pm standard error of the mean, $\times 10^4$: 174 ± 5 versus 427 ± 10 , $P < 0.001$). PreadSORption of immune sera with intact *S. sanguis* restored adherence to normal values, whereas preadsorption with dextran was partially effective. These studies demonstrate that preformed antibody has a protective role in vivo and suggest that a possible mechanism is blockade of adherence, a crucial early step in the pathogenesis of endocarditis.

Complex extracellular polysaccharide substances are important in the adherence of oral streptococci to dental surfaces (3, 8, 18) and the constituents of nonbacterial thrombotic endocarditis (14, 15) both in vitro and in vivo. This adherence may assume a critical role in the pathogenesis of two conditions: dental caries and infective endocarditis due to certain oral streptococci, especially *Streptococcus sanguis* and *Streptococcus mutans*. These observations have raised the possibility of prevention through immunization for these disorders. Protection has been achieved against dental caries in several animal systems by immunization against *S. mutans* (3, 8, 18). However, similar studies for infective endocarditis have yielded conflicting results.

Specific humoral antibody is usually assumed to offer a protective role against infection by the homologous organism. Paradoxically, in bacterial endocarditis, it has been accepted by many that the opposite may be true (5, 20). These conclusions were reached on the basis of four older reports published between 1919 and 1944 (10, 11, 19, 21). In these studies, endocarditis developed in variable proportions of horses and rabbits preimmunized with killed organisms after the injection of live pneumococci or meningococci. However, interpretation of the results of these investigations remains open to question,

since in two of the studies nonimmunized controls were not included (10, 19) and in the others (11, 21) endocarditis developed at rates approximately equivalent to those achieved in normal animals.

Opsonizing, agglutinating, and complement-fixing antibodies are present during active bacterial endocarditis (6, 7, 9, 13, 15). However, no study has focused on the importance of preformed antibody in the development of the disease. Therefore, the present experiments were first directed at determining the influence of preformed antibody on the development of endocarditis in vivo under controlled conditions. Durack and colleagues have reported recently that rabbits with high titers of complement-fixing antibodies after immunization with Formalin-killed *S. mutans* and *S. sanguis* were protected from development of endocarditis upon challenge with the homologous strain (2). Since a protective effect was confirmed in these studies, a possible mechanism was explored by analyzing the influence of preformed antibody on the adherence of *S. sanguis* to the constituents of nonbacterial thrombotic endocarditis in vitro. This adherence blockade hypothesis is analogous to the mechanism underlying the protection afforded by immunizations against dental caries (3, 8, 18).

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MATERIALS AND METHODS

Immunization protocol. A fresh isolate of the M-5 strain of *S. sanguis*, kindly provided by R. Gibbons, Forsyth Dental Institute, Boston, Mass., was utilized in these experiments. This organism has been employed previously in this laboratory in both in vitro adherence and in vivo rabbit endocarditis experiments (15). The organism was grown for 18 h at 37°C in brain heart infusion broth (Difco) supplemented with 5% sucrose and then centrifuged (3,000 rpm, 15 min) and resuspended in saline three times sequentially. The inoculum (2 ml) was alternately frozen and thawed five times in a dry ice-acetone bath and a 30 to 32°C water bath. Quantitative culturing on Trypticase soy agar (Difco) pour plates confirmed the absence of viable organisms after this procedure. The initial injection consisted of this preparation (0.25 ml) plus an equal volume of Freund complete adjuvant into both rear footpads of 2- to 3-kg New Zealand white rabbits. Subsequent injections of the same preparation were made subcutaneously every 5 days for 6 to 8 weeks. Precipitating antibody was then quantitated in serial dilutions of serum samples by double diffusion in agar against the autologous strain (see below). Immune sera contained anti-whole organism antibody in titers ranging from 1:2 to 1:16, with a median of 1:8. All preimmune sera were negative in this assay. Sera were also uniformly negative in six animals injected with Freund complete adjuvant alone.

Test for precipitating antibody. The antibody titers were determined by precipitation of antigen-antibody complexes by the application of standard double diffusion in agar (Ouchterlony method) for 24 h at 20°C. Antigen was prepared as described above, centrifuged (1,800 rpm, 30 min), and washed in saline before the wells were filled.

In vivo methods. Control rabbits or rabbits immunized against *S. sanguis* killed by freeze-thawing as previously described were catheterized by placement of a polyethylene catheter (Intramedic, Clay-Adams, PE-90) across the aortic valve. Thirty minutes of aortic valve trauma uniformly results in the development of nonbacterial thrombotic endocarditis (fibrin-platelet deposition). *S. sanguis*, after overnight incubation and resuspension in saline (1 ml), was injected at inocula varying from 10^5 to 10^8 colony-forming units (CFU) through the catheter, and the catheter was then removed. The animal was sacrificed at 48 h, and the vegetations were aseptically removed, weighed, homogenized, and quantitatively titrated onto duplicate Trypticase soy agar pour plates supplemented with 0.5% defibrinated sheep blood. Infectious dose-response curves were constructed and simplified by the techniques of probit analysis, as described previously (15).

In addition, six control animals and six immunized animals were catheterized as described, and 10^8 CFU of *S. sanguis* was injected through the catheter into the left ventricle. The catheter was then removed. Quantitative blood cultures (1-ml sample) were then

obtained from the central ear artery at 15-min intervals for 1 h, to assess bacterial clearance from the bloodstream. Two rabbits from each group were then sacrificed at 2, 4, and 6 h post-inoculation. The liver and spleen were removed aseptically from each animal, weighed, homogenized, and quantitatively titrated as above for the vegetations. Results were expressed as CFU per gram of wet tissue.

In vitro methods. The in vitro assay procedure for quantitation of bacterial adherence to the constituents of nonbacterial thrombotic endocarditis has been described previously and rigidly standardized (15). Briefly, three steps are required.

(i) Preparation of bacteria. An 18-h culture of a fresh isolate of *S. sanguis* M-5 (kindly supplied by R. Gibbons) in brain heart infusion broth containing 5% sucrose was centrifuged (3,000 rpm, 15 min) three times and sequentially resuspended in phosphate-buffered saline. The sample was filtered (8 μ m; Millipore Corp., Bedford, Mass.) to remove aggregated forms and then incubated with either preimmune or immune sera for 30 min at 37°C. During incubation, the mixture was vigorously blended in a Vortex mixer every 5 min for 15 s. After incubation the bacteria were refiltered (8 μ m) twice and verified as only single organisms by observations in a Petroff-Hauser chamber. The sample was recentrifuged (3,000 rpm, 15 min), washed twice in phosphate-buffered saline, and quantified by optical density measurements to provide an inoculum of 10^3 CFU (Gilford model 2400 spectrophotometer). In other experiments, immune sera were preabsorbed with either an 18-h culture of the homologous *S. sanguis* (1×10^8 to 3×10^8 CFU/ml for 15 min at 20°C) or purified extracellular dextran (2 mg/ml) from the homologous strain (15 min at 37°C), and the steps above were repeated. Dextran was prepared from 18-h cultures of *S. sanguis* M-5 in brain heart infusion broth plus 5% sucrose as described previously (15) by Sorvall disruption of cells and ethanol precipitation of extracellular polysaccharide.

(ii) Preparation of fibrin-platelet matrices. By differential centrifugation and filtration of anti-coagulated human blood, three suspensions were prepared with varying platelet concentrations, as previously described (15). These included platelet-rich plasma with a platelet count of 300,000/mm³, platelet-poor plasma (platelet count, 3,000/mm³), and platelet-free plasma (platelet count, 0/mm³). A 1-ml amount of platelet-rich, platelet-poor, or platelet-free plasma was combined with 0.4 ml of bovine thrombin (Thrombin, topical; 1,000 National Institutes of Health units per ml; Parke-Davis) at 500 U/ml and 0.4 ml of 0.2 M CaCl₂ in standard tissue culture dishes (60 by 15 mm; Falcon Plastics, Div. of Bioquest, Oxnard, Calif.) for 30 min at 37°C. Evenly spread adherent clots containing fibrin plus platelets in two concentrations (platelet-rich and platelet-poor plasmas) or fibrin alone (platelet-free plasma) were thus obtained and exposed to *S. sanguis*, as described below.

(iii) Determination of adherence. The inoculum of *S. sanguis* (2×10^3 to 7×10^3 CFU) in a volume of 5 ml of phosphate-buffered saline was exposed to the three surfaces for 15 min at 37°C in a shaking incubator (Dubnoff model 66799 metabolic incubator) oscillating at 120 cycles per min. Reproducible results

were obtained previously with this organism (15) when the tests were standardized for initial titer in the inoculum, time and temperature of incubation, volume of the inoculum, role of bacterial clumping, serum susceptibility, and specificity of adherence to the surface under study. After exposure, the supernatant was removed and quantitatively titrated. The supernatant contained less bacteria than the paired initial inoculum in all experiments, indicating that bacterial multiplication had not occurred during the 15-min exposure. The surfaces were then washed three times with phosphate-buffered saline, overlaid with Trypticase soy agar containing 0.5% defibrinated sheep blood, and incubated for 24 h at 37°C. The adherent organisms were then enumerated by colony count; the adherence ratio (AR) is defined as the number of organisms adherent to the surface (CFU) divided by the number of organisms in the initial inoculum (CFU). If the number of adherent organisms was divided by the number in the supernatant (CFU), all ARs were slightly higher, but relative differences remained identical. Because <1 to 5% of the organisms adhered under these test conditions, the AR was conventionally multiplied by 10^4 to facilitate comparisons. All statistical analyses were done on unpaired data with Student's *t* test.

Although bacterial clumping in serum was minimized by filtration and observing single unaggregated organisms in the standard bacterial suspensions, the following ratio was calculated in addition for all experiments: (number of bacteria in initial inoculum-number adherent to surface)/number of bacteria eluted in the supernatant after exposure. If all colonies on the surface result from adherence of a single organism, this ratio should equal 1.0. The actual mean \pm standard deviation for preimmune serum exposure experiments was 1.02 ± 0.02 ($n = 28$) versus 1.04 ± 0.02 ($n = 39$) after exposure to immune sera ($P = 0.76$). This confirms that virtually every colony results from a single adherent organism and excludes bacterial aggregation during the experimental procedure.

RESULTS

In vivo. Immunization of rabbits with *S. sanguis* killed by freeze-thawing protected the animals from the development of endocarditis. The infectious dose (inoculum required to produce endocarditis in 50% of the animals) was $10^{6.5 \pm 0.33}$ (mean \pm standard deviation) for 30 control rabbits. However, in 36 immunized rabbits the mean infectious dose was increased by over 1 log, to $10^{7.71 \pm 0.05}$ ($P < 0.01$). Expressed another way, when 10^7 *S. sanguis* cells were injected (an inoculum between the two mean infectious doses), 6 of 9 control animals developed endocarditis, compared with 1 to 10 immunized rabbits ($P = 0.03$, Fischer's exact test). Thus, immunization prevented, rather than potentiated, the development of endocarditis in this experimental model. Although the numbers were too small for statistical comparison, animals with the highest antibody titers had the lowest vegetation bacterial titers.

This protective effect of antibody could not be explained by an accelerated rate of bacterial clearance from the bloodstream in the immunized animals. The clearance of bacteria (as reflected by bacterial titer per milliliter of blood over time) was rapid and virtually identical for the two groups for 1 h postinjection (Fig. 1). After 2 h, the blood culture titers continually increased for the control group, as opposed to a steady decline in the immunized animals, reflecting the development of endocarditis in the controls. In addition, no differences were seen in liver or spleen bacterial titers between the two groups when they were sampled 2, 4, and 6 h postinjection.

In vitro. Homologous immune sera decreased the adherence of *S. sanguis* to the constituents of nonbacterial thrombotic endocarditis in vitro (Table 1). *S. sanguis* preincubated in control sera exhibited the highest adherence ($\times 10^4$) for the platelet-rich plasma surface (427 ± 10). When *S. sanguis* was preincubated with immune sera, the AR was consistently reduced. Adherence blockade was apparent even at low antibody titers of 1:2, as demonstrated by a reduction in the mean AR ($\times 10^4$) to the platelet-rich plasma matrix from 427 ± 10 to 253 ± 4 ($P < 0.001$). Antibody titers of 1:4 had ARs similar to those observed at 1:2, but preincubation of the organism in immune sera with antibody titers of 1:8 produced a further reduction in adherence to the lowest levels observed (174 ± 3 , $P < 0.001$). A similar reduction in adherence with organisms preincubated in antibody-containing sera was observed for fibrin monolayers without platelets (Table 1).

Prior adsorption of immune sera with *S. sanguis* killed by freeze-thawing (initial titer, 10^8 CFU/ml) eliminated detection of precipitating antibody ($\geq 1:8$ to 0) and completely nullified the adherence blocking effect of the serum. In fact, the AR was increased (460 ± 11) in adsorbed

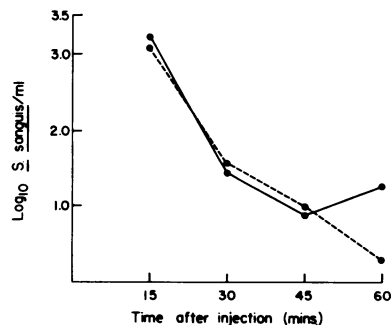


FIG. 1. Bacterial titer (CFU per milliliter of blood) versus time after injection. Symbols: ●—●, control animals ($n = 6$); ●—●, immunized animals ($n = 6$).

TABLE 1. Relationship of antibody titer to adherence of *S. sanguis* in vitro

Antibody titer	Treatment	AR ($\times 10^4$)		
		PRP ^a	PPP ^a	PPF ^a
0 (controls; $n = 14$)		427 \pm 10 ^b	498 \pm 6	260 \pm 10
1:2 ($n = 16$)		253 \pm 4	243 \pm 5	151 \pm 8
1:4 ($n = 10$)		257 \pm 7	203 \pm 6	144 \pm 6
1:8 ($n = 20$)		174 \pm 5	137 \pm 3	97 \pm 11
1:8 ($n = 8$)	Preadsorbed with <i>S. sanguis</i>	460 \pm 11	ND ^c	ND
1:8 ($n = 6$)	Preadsorbed with dextran	269 \pm 6	ND	ND

^a PRP, Platelet-rich plasma; PPP, platelet-poor plasma; PFP, platelet-free plasma.

^b Mean \pm standard error of the mean.

^c ND, Not done.

sera compared with nonadsorbed sera from non-immunized controls (427 \pm 10, $P < 0.05$). Adsorption with purified *S. sanguis* extracellular polysaccharide (dextran) at 2 mg/ml also reduced the adherence-blocking effect of immune sera but did so less effectively than the whole organism; the AR increased from 174 \pm 5 to 269 \pm 6 ($P < 0.01$). Thus, anti-whole organism antibody blocks the adherence of *S. sanguis* to the constituents of nonbacterial thrombotic endocarditis, an effect partially dependent on antibody concentration.

DISCUSSION

This study demonstrates that preformed homologous antibody protects rabbits from the development of *S. sanguis* endocarditis in vivo. Immunized rabbits did not exhibit an enhanced clearance of bacteria from the blood, but immune sera were effective in blocking the adherence of *S. sanguis* to the constituents of nonbacterial thrombotic endocarditis (fibrin and platelets) in vitro. This suggests that the protective mechanism(s) may be antibody blockade of early attachment or colonization of the damaged valve in vivo.

These results conflict with the currently widely accepted paradoxical view (20) that preformed humoral antibody potentiates endocarditis development. This view arose from several studies. Wadsworth (19) noticed that seven of eight horses previously immunized with pneumococci developed endocarditis after injection of live pneumococci. However, nonimmunized controls were not included, all blood cultures were negative, and huge inocula in potentially pathogenic broth were injected directly. Mair (10) produced similar results in 70% of rabbits, but again controls were not included. These results could not be confirmed by Wright (21), who found that only 6 of 58 rabbits developed endocarditis under similar immunizing conditions. The results were "fortuitous" and "unpredictable" and likely represented interanimal var-

iation (21). Miller (11) noted endocarditis in only 14 of 110 horses previously immunized against meningococci upon injection of the homologous strain. It should be emphasized that these endocarditis rates approximate those found in non-immunized animals after the injection of viable bacteria. These early investigators also injected organisms in broth directly, and clumps of material were often present (21). Particulate matter may cause reticuloendothelial blockade and increase endocarditis susceptibility by this mechanism (16). Thus, critical review of these earlier studies does not permit any conclusions regarding the role of preformed antibody in this disease.

Opsonic, agglutinating, and complement-fixing antibodies of both immunoglobulin G and immunoglobulin M classes have long been recognized during bacterial endocarditis (6, 7, 9, 13, 17). Titers increase with the duration of symptoms and decrease with effective therapy (13). These antibodies are likely a response to infection and not preformed (17) since they are often absent early in the bacteremic stage and appear with clearance of organisms from the blood. A similar time course is suggested for circulating antigen-antibody immune complexes in this disease (1). Virtually 100% of cases of viridans streptococcal endocarditis demonstrate homologous humoral immunity (6, 17), but none of these studies addresses the influence of antibody on the early events necessary for the development of endocarditis.

Oral viridans streptococci still cause the majority of cases of bacterial endocarditis, and *S. sanguis* is the most common of these isolates (4). Most strains, including *S. sanguis* and *S. mutans* strains, produce complex extracellular polysaccharides, called dextrans, which facilitate adherence to tooth surfaces and are important factors in the pathogenesis of dental caries. These substances also are important in the pathogenesis of infective endocarditis, as shown previously (15). Immunization of animals against *S.*

mutans is protective against dental caries formation (3, 8, 18), and it has been suggested that future employment of a dental caries vaccine in humans might paradoxically increase the risk of endocarditis in the recipients for the reasons discussed above. This study and the recent report of Durack and colleagues (2) suggest that this would not occur and that immunization is, in fact, protective against the development of infective endocarditis. This study further suggests that the protective mechanism may be mediated by adherence blockade, a crucial early step in the pathogenesis of endocarditis. Although immunization against infective endocarditis may be impractical because of the wide variety of causative organisms, common adherence factors may exist between organisms, and further study is needed to explore these possibilities. The specific factors (antigens) that the adherence-blocking antibodies were directed against were not completely delineated in this study. Adsorption of serum with whole organisms completely eliminated the adherence blockade and actually resulted in higher ARs than nonadsorbed control rabbit sera. This suggests that adherence-blocking antibody also existed in control sera but at low levels that were not detected by the common diffusion technique employed. Adsorption of sera with a single concentration of dextran precipitated from a culture of the test organism markedly reduced but did not completely eliminate the adherence blockade. Thus, although cell surface dextrans clearly influence adherence of *S. sanguis* to fibrin and platelets and the development of endocarditis (12, 14, 15), other cell surface antigens may also play a critical role in this event.

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